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<b>(54) Title:</b> A BIOLUMINESCENCE RESONANCE ENERGY TRANSFER (BRET) SYSTEM AND ITS USE  <b>(57) Abstract</b>  This invention provides bioluminescence resonance energy transfer (BRET) system that comprises four parts: 1) a bioluminescent protein that has luciferase activity; 2) an acceptor fluorophore that can accept the energy from the bioluminescent protein when they are associated, in the presence of the appropriate substrate; 3) a modulator that influences the proximity or the orientation of the bioluminescent protein and the fluorophore, and 4) an appropriate substrate to activate the luciferase activity of the bioluminescent protein. The components of this system interact to influence the spatial relationship between the bioluminescent protein and the fluorophore, that is demonstrated by the light emission from the system. The modulator can be a single entity, covalently attached to both the bioluminescent protein and the fluorophore, it can be two separate entities, each linked covalently to either the bioluminescent protein or the fluorophore, or an alternative configuration that falls within the scope of the invention. This system can be used in both <i>in vivo</i> or <i>in vitro</i> assays to detect molecular changes in a wide variety of applications, and is amenable to automation. In particular, it is useful for assaying protein interactions, enzyme activities and the concentration of analytes or signaling molecules in cells or in solution.		

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## **A BIOLUMINESCENCE RESONANCE ENERGY TRANSFER (BRET) SYSTEM AND ITS USE**

### **FIELD OF THE INVENTION**

This invention pertains to the field of molecular interactions.

### **BACKGROUND OF THE INVENTION**

Interactions between proteins and other molecules play a key regulatory role in almost every biological process. Thus, many techniques have been developed to identify and characterize these interactions. These tools range from in vitro binding assays to library-based methods and include genetic methods such as searching for extragenic suppressors (Phizicky, E. M. & Fields, S., (1995) *Microbiol. Rev.*, **59**, 94-123). Currently, the most popular library-based method is the yeast two-hybrid system, which allows the selection of yeast strains that contain two interacting peptides, the target peptide and another from the library (Fields, S. & Song, O. K., (1989) *Nature (London)* **340**, 245-246). This method has proven itself to be a valuable technique for identifying potential protein interactions, but it has several liabilities, foremost of which is that the interaction is tested only in the nucleus of a yeast cell. This means interactions that depend upon cell-type specific processing and/or compartmentalization will not be detected by the two-hybrid system.

Another technique for assessing protein-protein interaction is based on fluorescence resonance energy transfer (FRET). In this process, one fluorophore (the "donor") transfers its excited-state energy to another fluorophore (the "acceptor") which usually emits fluorescence of a different color. FRET efficiency depends on three parameters: (i) the overlap between the absorption spectrum of the second fluorophore and the emission spectrum of the first fluorophore, (ii) the relative orientation between the emission dipole of the donor and the absorption dipole of the acceptor, and (iii) the distance between the fluorophores. Depending on the spectral overlap and dipole orientations, the distance over which FRET occurs can be between 10-100 Å (Wu, P. & Brand, L., (1994) *Anal. Biochem.*, **218**, 1-13). FRET has been used to assay protein-protein proximity in vitro and in vivo by chemically attaching fluorophores such as fluorescein and rhodamine to pairs of purified proteins and measuring fluorescence

spectra of protein mixtures or cells that were microinjected with the labeled proteins (REF). The cloning and expression of Green Fluorescent Protein (GFP) in heterologous systems opened the possibility of genetic attachment of fluorophores to proteins. In addition, the availability of GFP mutants with altered wavelengths (Heim *et al.*, (1994) *Proc. Natl. Acad. Sci. USA.*, 91, 12501-12504) allowed their use as FRET pairs. For example, GFP and its blue-fluorescent mutant, BFP were first used to demonstrate FRET between GFP molecules (Heim, R. & Tsien, R. Y., (1996) *Curr. Biol.*, 6, 178-182). Other GFP pairs such as the cyan CFP, and yellow-green YFP considerably extended the applications in which FRET can be used to measure protein-protein interactions (Miyawaki *et al.*, (1997) *Nature (London)*, 388, 882-887).

An attractive application allowed by GFP-based FRET is the in vivo assay of protein interactions in organisms other than yeast. For example, fusion of GFP and BFP to the mammalian transcriptional factor Pit-1, showed homo-dimerization of Pit-1 in live HeLa cells (Periasamy, A. and Day, R. N., (1998) *J. Biomed. Opt.*, 3, 1-7). In this type of assay, interactions can be examined in the proteins' native organism, such that cell-type specific modifications and/or compartmentalization of the proteins are preserved. Additionally, compartmentalization of these interacting proteins is potentially visible in the microscope. FRET, however, has several limitations. As with any fluorescence technique, photobleaching of the fluorophore and autofluorescence of the cells/tissue can significantly restrict the usefulness of FRET, and in highly autofluorescent tissues, FRET is essentially unusable. Also, if the tissue is easily damaged by the excitation light, the technique may be unable to give a value for healthy cells. Finally, if the cells/tissues to be tested are photoresponsive (e.g., retina), FRET may be impractical because as soon as a measurement is taken, the photoresponse may be triggered.

## SUMMARY OF THE INVENTION

This invention provides a bioluminescence resonance energy transfer (BRET) system that comprises four parts: 1) a bioluminescent protein that has luciferase activity; 2) an acceptor fluorophore that can accept the energy from the bioluminescent protein when they are associated, in the presence of the appropriate substrate; 3) a modulator that influences the proximity or the orientation of the bioluminescent protein

and the fluorophore, and 4) an appropriate substrate to activate the luciferase activity of the bioluminescent protein. The components of this system interact to influence the spatial relationship between the bioluminescent protein and the fluorophore, that is demonstrated by the light emission from the system.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a pictorial description of a BRET system wherein the luciferase and the fluorophore are attached to different proteins or peptides.

Figure 2 is a pictorial description of a BRET wherein the luciferase and the fluorophore are attached to the same protein or peptide.

Figure 3 shows *in vivo* bioluminescence resonance energy transfer (BRET) of RLUC in *E. coli* cells expressing the RLUC•EYFP fusion protein. (a) Diagrams of the expression cassettes of pT7/Eyfp, pT7/Rluc, and pT7/Rluc•Eyfp. Abbreviations: P<sub>T7</sub> = T7 promoter region; Ter = transcription terminator region. Antibiotic resistances are indicated within parentheses. The linker sequence between Rluc and Eyfp in the Rluc•Eyfp fusion construct is shown below the pT7/Rluc•Eyfp diagram. (b) Luminescence (lum.) or fluorescence (fluor.) emission spectra from transformed strains expressing the proteins RLUC or EYFP or the fusion protein RLUC•EYFP. All spectra were normalized.

Figure 4 demonstrates an *in vivo* BRET assay for circadian clock protein interaction. (a) Diagrams of the gene fusion expression cassettes of pT7/Eyfp•kaiB, pT7/Rluc•kaiB, and pT7/Eyfp•kaiA. (b) Luminescence emission spectra from the transformed strains expressing RLUC & EYFP (b1), RLUC•KaiB & EYFP•KaiB (b2), and RLUC•KaiB & EYFP•KaiA (b3).

Figure 5 shows clock protein KaiB-KaiB association *in vitro* revealed by BRET. Extracts containing fusion proteins EYFP•KaiB were mixed in equal proportions with those containing RLUC•KaiB. Bioluminescence emission profiles were measured at the indicated times after the extracts were combined and incubated at room temperature.

Figure 6 demonstrates imaging of bioluminescence from transformed *E. coli* strains through interference bandpass filters transmitting light of  $480 \pm 5$  nm (left panels) or  $530 \pm 5$  nm (right panels). Row (a) is the strain producing only RLUC; row (b) is the strain expressing the RLUC EYFP fusion protein; row (c) is the strain expressing the fusion proteins RLUC•KaiB and EYFP•KaiA; and row (d) is the strain producing the fusion proteins RLUC•KaiB and EYFP•KaiB. (Blue color is pseudo-color indicating the intensity of the light emission; it does not indicate the actual color of the light emitted).

Figure 7 shows quantification of BRET intensity and ratios in the images from Figure 6. Upper panel, quantification of luminescence intensity (in relative light units) at 480 nm and 530 nm of the cultures imaged in Figure 6. Bottom panel, calculation of the 530 nm/480 nm ratios. Strains are labelled in Figure 6: (a) RLUC, (b) RLUC•EYFP, (c) RLUC•KaiB and EYFP•KaiA, and (d) RLUC•KaiB and EYFP•KaiB.

Figure 8 shows the results of an in vitro enterokinase assay, demonstrating that only the ratio for the construct Rluc:enterokinase:EYFP decreases over time in the presence of the enterokinase enzyme.

Figure 9 represents the timecourse of interaction of the different kai proteins at 25°C. Only the ratio for the Rluc-kaiB EYFP-kaiB increase over time for both transfection condition (using 4 or 0.5mg DNA per vector). Ratios for all the negative controls Rluc-kaiB and Rluc-kaiB +EYFP-kaiA, do not increase with time. This means that energy transfer occurs only for the Rluc-kaiB EYFP-kaiB which is as expected since kaiB bind only with itself and not with kaiA.

Figure 10 depicts a timecourse of induction by calcitonin and forskolin.

Figure 11 shows a dose response of calcitonin using CHO-K1 cell line.

Figure 12 shows a timecourse of induction of  $\beta_2$  receptor by isoproterenol.

Figure 13 shows a dose response of isoproterenol on the cells transfected with cAMP sensor CBP:CREB. Data were taken at 45 minutes after addition of the isoproterenol and the coelenterazine.

The assay was performed at 25°C.

Figure 14 demonstrates the results of a timecourse experiment on the activation of CCR5 receptor using forskolin and MIP-1b, a selective agonist for CCR5.

Figure 15 demonstrates the results of a time course experiment on the activation of CCR5 receptor using forskolin and MIP-1b.

Figure 16 shows an EGF dose response on the GH4-C1 cells transfected with the TKR sensor.

Figure 17 depicts a timecourse experiment on cells transfected with the different b2 constructs.

Figure 18 shows a comparison of the BRET ratio at 20 minutes after addition of coelenterazine.

Figure 19 presents the results of experiments demonstrating the kinetics of b2 dimerization.

Figure 20 shows a dose response analysis.

Figure 21 shows the results of a BRET assay performed using the BRETCount.

Figure 22 presents a spectral analysis of BRET reaction at different time points after coelenterazine addition using the Spex. The Y axis represents the CPS and X axis represents the wavelength.

Figure 23 demonstrates the effect of cell number per well.

Figure 24 shows the results of experiments determining Rluc luminescence in PBS Ca<sup>2+</sup>/Mg<sup>2+</sup> in the presence or absence of DTT.

Figure 25 shows the results of experiments determining Rluc luminescence in PBS Ca<sup>2+</sup>/Mg<sup>2+</sup> + glucose in the presence or absence of DTT.

Figure 26 shows the results of experiments determining Rluc luminescence in DMEM without phenol red in the presence or absence of DTT.

Figure 27 demonstrates the effect of pH on the BRET ratio.

Figure 28 shows the effect of Hepes-based buffer on Rluc construct ratio

Figure 29 shows the effect of Hepes-based buffer on Rluc::EYFP construct ratio

Figure 30 shows the effect of DMSO on the energy transfer.

Figure 31 shows apoptosis induction using staurosporine in HeLa cells transfected with the sensor.

Figure 32 shows results from the MiniScreen Assay

Figure 33 presents the results of an experiment demonstrating a calcitonin dose response on CHO-K1 cell transfected with the cAMP sensor (CBP:CREB constructs).

Figure 34 shows an isoproterenol dose response on CHW- $\beta$ 2 cell transfected with the cAMP sensor (CBP:CREB constructs). In this case, the ratio has been corrected using the algorithm formula presented in Example XIV.

Figure 35 presents results from a cell linearity experiment.

Figure 36 shows results from a time-course study of Rluc luminescence.

Figure 37 shows a dose response of colelenterazine derivatives.

Figure 38 presents results from a study comparing lysed and non-lysed cells.



Figure 39 shows LucLite stabilization of Rluc luminescence in live cells.

Figure 40 presents results of a ratio stability study when using coelenterazine h in LucLite.

Figure 41 shows results of a ratio stability study when using native coelenterazine in LucLite.

Figure 42 shows BRET detection in cells transfected with 10 times less DNA.

Figure 43 presents results demonstrating BRET detection in cells transfected with 20 times less DNA.

Figure 44 presents results of a study of BRET detection when cells are lysed. Transfections were done using the "normal" amount of DNA.

Figure 45 presents results of a comparison study between PBS and DMEM/F12 (without phenol red) for assaying BRET.

Figure 46 shows DTT effects on the Rluc emission stability.

Figure 47 demonstrates Rluc emission stability over time.

Figure 48 shows Rluc emission levels vs. cell number at fixed time.

Figure 49 presents Rluc emission stability in the presence of a fixed number of cells.

Figure 50 shows a demonstration of Rluc emission levels vs. cell number at fixed times.

Figure 51 shows a demonstration of the effect of temperature on the Rluc emission.

Figure 52 shows a demonstration of the effect of the temperature on the BRET ratio (LumiCount).

Figure 53 shows a demonstration of the effect of the temperature on the BRET ratio (BRETCount).

Figure 54 shows a demonstration of the effect of DTT on the Rluc emission.

Figure 55 shows a demonstration of the effect of DTT on the Rluc emission. And the BRET ratio (BRETCount). These results were obtained with the stable cell lines expressing Rluc only (negative control).

Figure 56 shows a demonstration of the effect of DTT on the Rluc emission. And the BRET ratio (BRETCount). These results were obtained with the stable cell lines expressing the fusion Rluc::EYFP (positive control).

Figure 57 shows the DNA sequence for the Rluc-EYFP construct.

Figure 58 shows the DNA sequence for the Rluc-enterokinase-EYFP construct.

Figure 59 shows the DNA sequence for the Rluc-caspase-EYFP construct.

Figure 60 shows the DNA sequence for the Rlu-CREB construct.

Figure 61 shows the DNA sequence for the Rlu-CBP construct.

Figure 62 shows the DNA sequence for the EYFP-SH2 construct.

Figure 63 shows the DNA sequence for the Rluc-PTB construct.

## **DETAILED DESCRIPTION OF THE INVENTION**

### **Definitions**

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in spectroscopy, drug discovery, cell culture, molecular genetics, plastic manufacture, polymer chemistry, diagnostics, amino acid and nucleic acid chemistry, and sugar chemistry described below are those well known and commonly employed in the art. Standard techniques are typically used for preparation of plastics, signal detection, recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection).

The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., and Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, New York: Plenum Press (1983) for fluorescence techniques, which are incorporated herein by reference) which are provided throughout this document. Standard techniques are used for chemical syntheses, chemical analyses, and biological assays. As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

“Bioluminescent protein” refers to a form of chemiluminescence which arises as the result of an energy-yielding chemical reaction in which a specific biochemical substance, a luciferin (a naturally occurring fluorophore), is oxidized by an enzyme having a luciferase activity. A great diversity of organisms, both prokaryotic and eukaryotic, including species of bacteria, algae, fungi, insects, fish and other marine forms can emit light energy in this manner and each has specific luciferase activities and luciferins which are chemically distinct from those of other organisms. Luciferin/luciferase systems are very diverse in form, chemistry and function. For example, there are luciferase activities which facilitate continuous chemiluminescence, as exhibited by some bacteria and mushrooms, and those which are adapted to facilitate sporadic or stimuli induced emissions, as in the case of dinoflagellate algae. As a phenomenon which entails the transformation of chemical energy into light energy, bioluminescence is not restricted to living organisms, nor does it require the presence of living organisms. It is simply a type of chemiluminescent reaction that requires a luciferase activity which at one stage or another had its origins

from a biological catalyst. Hence the preservation or construction of the essential activities and chemicals suffices to have the means to give rise to bioluminescent phenomena. Bioluminescent proteins with luciferase activity are thus available from a variety of sources or by a variety of means. Examples of bioluminescent proteins with luciferase activity may be found in U.S. Patent Nos 52229285, 5219737, 5843746, 5196524, 5670356.

“Fluorophore” refers to any molecule which can accept energy from the bioluminescent protein with luciferase activity, and re-emit it as light energy. There are a number of different fluorophores that can be employed in this invention. One very well known group of fluorophores includes the green fluorescent protein from the jellyfish *Aequorea victoria* and numerous other variants (GFPs) arising from the application of molecular biology, eg. mutagenesis and chimeric protein technologies (R.Y. Tsien, (1998) *Ann. Rev. Biochem.* 63: 509-544). GFPs are classified based on the distinctive component of their chromophores, each class having distinct excitation and emission wavelengths: class 1, wild-type mixture of neutral phenol and anionic phenolate: class 2, phenolate anion: class 3, neutral phenol: class 4, phenolate anion with stacked  $\pi$ -electron system: class 5, indole: class 6, imidazole: and class 7, phenyl (R.Y. Tsien, (1998), *supra*).

GFPs or other fluorophores, bioluminescent proteins with luciferase activities and the appropriate substrates have been combined into luminescence based assay, tagging, screening and protein-protein interaction detection systems: for example see U.S. Patent Nos. 5786162, 4614712, 5650289, 5837465, 5897990, 57002883, 54016229, 5221623, 5854010, 5770391, 4665022, 5854004.

“Substrate” refers to any means for activating the bioluminescent protein with luciferase activity. In a preferred embodiment *Renilla* luciferase (Rluc) (Cormier, (1978), *Methods Enzymol.*, 57, 237-244), or one of its derivatives is used as the donor fluorophore because its emission spectrum is similar to the cyan ECFP (l max Å 480 nm) which has been shown to exhibit FRET with EYFP (Miyawaki *et al.*, (1997) *Nature (London)* 388, 882-887). The coelenterazine substrate for RLUC is a hydrophobic molecule that is permeable to most cell types.

“Protein” refers to include a whole protein, or fragment thereof, such as a protein domain or a binding

site for a second messenger, co-factor, ion, etc. It can be a peptide or an amino acid sequence that functions as a signal for another protein in the system, such as a proteolytic cleavage site.

"Functional genomics" refers to the field pertaining to the determination of cellular function of a new gene. One manner of determining the function of a protein is to determine the identity of its binding partner. One application of the BRET system is to enable the identification of a gene product, or its target location within a cell, or the proteins, nucleic acid sequences, or other biomolecules with which it interacts.

"Binding pair" refers to two moieties (e.g. chemical or biochemical) that have an affinity for one another. Examples of binding pairs include homo-dimers, hetero-dimers, antigen/antibodies, lectin/avidin, target polynucleotide/probe, oligonucleotide, antibody/anti-antibody, receptor/ligand, enzyme/ligand and the like "One member of a binding pair" refers to one moiety of the pair, such as an antigen or ligand.

"Membrane-permeant derivative" refers a chemical derivative of a compound that has enhanced membrane permeability compared to an underivatized compound. Examples include ester, ether and carbamate derivatives. These derivatives are made better able to cross cell membranes, i.e. membrane permeant, because hydrophilic groups are masked to provide more hydrophobic derivatives. Also, masking groups are designed to be cleaved from a precursor (e.g., fluorogenic substrate precursor) within the cell to generate the derived substrate intracellularly. Because the substrate is more hydrophilic than the membrane permeant derivative it is now trapped within the cells.

"Modulator" means a molecule or molecules that will undergo a conformational change in response to an interaction with another molecule, thereby affecting the proximity and/or orientation of the bioluminescent protein and the fluorophore. A modulator can be peptide, protein, nucleic acid or other synthetic or natural molecule. Examples of modulators for this invention include, a protease site, a second messenger binding site, an ion binding molecule, a homo-dimer or hetero-dimer molecule, a nucleic acid sequence, a kinase substrate or other enzyme substrate, a receptor or a receptor ligand.

"Isolated polynucleotide" refers a polynucleotide of genomic, cDNA, or synthetic origin or some

combination thereof, which by virtue of its origin the "isolated polynucleotide" (1) is not associated with the cell in which the "isolated polynucleotide" is found in nature, or (2) is operably linked to a polynucleotide which it is not linked to in nature.

"Isolated protein" refers a protein of cDNA, recombinant RNA, or synthetic origin or some combination thereof, which by virtue of its origin the "isolated protein" (1) is not associated with proteins found it is normally found with in nature, or (2) is isolated from the cell in which it normally occurs or (3) is isolated free of other proteins from the same cellular source, e.g. free of human proteins, or (4) is expressed by a cell from a different species, or (5) does not occur in nature. "Isolated naturally occurring protein" refers to a protein which by virtue of its origin the "isolated naturally occurring protein" (1) is not associated with proteins that it is normally found with in nature, or (2) is isolated from the cell in which it normally occurs or (3) is isolated free of other proteins from the same cellular source, e.g. free of human proteins.

"Polypeptide" as used herein as a generic term to refer to native protein, fragments, or analogs of a polypeptide sequence. Hence, native protein, fragments, and analogs are species of the polypeptide genus.

"Naturally-occurring" as used herein, as applied to an object, refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

"Control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding and non-coding sequences to which they are ligated. The nature of such control sequences differs

depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

"Polynucleotide" refers to a polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA.

"Corresponds to" refers to a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

"Polypeptide fragment" refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion, but where the remaining amino acid sequence is usually identical to the corresponding positions in the naturally-occurring sequence deduced, for example, from a full-length cDNA sequence. Fragments typically are at least 5, 6, 8 or 10 amino acids long, preferably at least 14 amino acids long, more preferably at least 20 amino acids long, usually at least 50 amino acids long, and even more preferably at least 70 amino acids long.

"Plate" refers to a multi-well plate, unless otherwise modified in the context of its.

The term "test chemical" refers to a chemical to be tested by one or more screening method(s) of the invention as a putative candidate.

The terms "label" or "labeled" refers to incorporation of a detectable marker, e.g., by incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods). Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes (e.g.,  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (or reporter genes) (e.g., horseradish peroxidase,  $\beta$ -galactosidase,  $\beta$ -lactamase, luciferase, alkaline phosphatase), chemiluminescent, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

"Genotoxicity" refers to the effect of a toxic compound on the stability of genes or genomes.

Common examples of second messengers include cAMP, cGMP,  $\text{Ca}^{2+}$ , IP3, NO, DAG, ceramide and derivatives thereof, and arachidonic acid and derivatives thereof.

The terms, analyte, refers to a substance for which the presence, absence, or quantity is to be determined. An analyte is typically a small molecule or ionic solute such as  $\text{K}^+$ ,  $\text{H}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{CO}_2$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{Mg}^{2+}$ ,  $\text{O}_2$ ,  $\text{HCO}_3^-$ , NO, and ATP.

As is evidenced throughout the text, some terms are used interchangeably to denote equivalent concepts. For example, the term "sensor" is used to denote a modulator entity that performs a specific function.

Common examples of ion channels include  $\text{K}^+$ -coupled receptor,  $\text{Ca}^{2+}$ -channels,  $\text{K}^+$ -voltage-sensitive channels,  $\text{K}^+$ -channel  $\text{Ca}^{2+}$  sensitive

Other chemistry terms herein are used according to conventional usage in the art, as exemplified by The



McGraw-Hill Dictionary of Chemical Terms (ed. Parker, S., 1985), McGraw-Hill, San Francisco, incorporated herein by reference).

#### Embodiments of the Invention

This invention provides a bioluminescence resonance energy transfer (BRET) system that comprises four parts: 1) a bioluminescent protein that has luciferase activity; 2) an acceptor fluorophore that can accept the energy from the bioluminescent protein when they are associated, in the presence of the appropriate substrate; 3) a modulator that influences the proximity or the orientation of the bioluminescent protein and the fluorophore, and 4) an appropriate substrate to activate the luciferase activity of the bioluminescent protein. The components of this system interact to influence the spatial relationship between the bioluminescent protein and the fluorophore, that is demonstrated by the light emission from the system. The modulator can be a single entity, covalently attached to both the bioluminescent protein and the fluorophore as demonstrated in Figure 1, it can be two separate entities, each linked covalently to either the bioluminescent protein or the fluorophore as demonstrated in Figure 2, or an alternative configuration that falls within the scope of the invention. This system can be used in both *in vivo* or *in vitro* assays to detect molecular changes in a wide variety of applications, and is amenable to automation. In particular, it is useful for assaying protein interactions, enzyme activities and the concentration of analytes or signaling molecules in cells or in solution. It is useful for drug discovery, analyte screening, second messenger screening, drug screening, diagnosis, genotoxicity, identification of gene function, gene discovery, and proteomics.

One preferred embodiment of this system is exemplified in Figure 1. In this embodiment, there are generally two entities comprising the modulator, wherein the bioluminescent protein is covalently attached to one modulator entity and the fluorophore is covalently attached to the second modulator entity. The interaction between the two modulator entities determines the proximity and orientation between the bioluminescent protein and the fluorophore, and therefore the light emission from the system.

This embodiment can be used to detect the interaction (moving together, moving apart, or changing their

orientation to one another) of the two modulator entities. In one example, wherein this embodiment can be used for detecting binding partners, the bioluminescent protein can be attached to one gene product and the fluorophore can be attached to an unknown gene product, such that interaction between the two gene products will be displayed by the light emission of the fluorophore. In another application, such as drug discovery, this embodiment is used to determine if a chemical or molecular substance will cause the molecular entities such as homo-dimers or hetero-dimers to move together or to move apart. By attaching the bioluminescent protein to one of the dimer-proteins and the fluorophore to the other dimer-protein, changes in the spatial relationship between the dimer-proteins in response to a chemical compound such as a drug candidate, will translate to the spatial relationship between the bioluminescent protein and the fluorophore, that will be signaled by a change the light emission from the system.

Another preferred embodiment is exemplified in Figure 2. In this embodiment, the bioluminescent protein and the fluorophore are attached to a common linker comprising the modulator component of the system. One typical example of this embodiment is the use of the modulator as a sensor that will detect changes in a component in the environment, causing the sensor to undergo a conformational change that affects the spatial relationship of the bioluminescent protein and the fluorophore, that is signalled by the light emission from the system.

This bioluminescent protein-modulator-fluorophore construct (BMFC) can be produced in a number of ways. It can be produced entirely using recombinant methods by preparing and expressing a recombinant DNA encoding the BMFC fusion protein. Alternatively, it can be produced by isolating the component proteins from a recombinant system and then covalently attaching the units using chemical means. Alternatively, the fusion protein construct can be produced in two parts, wherein the bioluminescent protein and the fluorophore are produced using recombinant methods, optionally fused to a peptide linker or a portion of the modulator component. In the latter situation, all or part of the modulator can be attached chemically to each peptide linker or directly to the bioluminescent protein or fluorophore, thereby generating a linear construct with the bioluminescent protein joined to the fluorophore through the peptide linker comprising the modulator. The modulator can be a single entity or two entities that interact. The modulator can further be either biologically derived or chemically synthesized or a modified biological molecule. Another option is to isolate the bioluminescent protein

and/or the fluorophore from natural sources and chemically join them to the modulator.

There are a wide variety of examples demonstrating the versatility and usefulness of the BRET system.

For example, a bioluminescent protein-modulator-fluorophore construct (BMFC) is presented in Example IV

*The Use of the BRET Assay of Protein-Protein Interactions for in vivo Assays*

The BRET system is useful for *in vivo* assays. In general, the proteins will be introduced by transforming or transfecting a cell with one or more vectors comprising the recombinant DNA encoding some of the components of the system. The cell will produce the protein and BRET will occur when the luciferase, the fluorophore and the substrate are in the appropriate spatial relationship. The substrate can be either added or introduced in its nucleic acid form, such as via a vector with an inducible promoter.

*The Use of the BRET Assay of Protein-Protein Interactions for in vitro Assays*

The components of the BRET system can be produced using molecular biology techniques or isolated from natural sources. After purification, they can be used in non-cell based in vitro assays.

One application of this system to an in vitro system is demonstrated by the results presented in Figure 3, which presents spectra derived from mixing *E. coli* extracts from two different cultures, one expressing RLUC•KaiB and the other expressing EYFP•KaiB. In the luminescence spectrum acquired immediately after mixing, there is no evidence for a second peak. After room temperature incubation for 30 minutes, a significant shoulder has emerged on the spectrum of the mixed extracts. At the 30 min incubation time point, the intensity of the RLUC luminescence has also decreased to approximately 60% of the time 0 value. This decrease in luminescence intensity is likely due to proteolysis within the extract. After a two hours of room temperature incubation, the luminescence exhibited a clear bimodal spectrum, although the overall luminescence intensity was only about 6% of

the initial value. Even though the assay needs further optimization, the development of a shoulder followed by an obvious bimodality indicates that BRET can be assayed *in vitro*.

#### The Use of the BRET Assay for High-Throughput Screening

As demonstrated in Example XI, the BRET system is particularly amenable to automation and high-throughput screening.

This example also demonstrates that the BRET system can be useful for *in vivo* assays. For example, the ratio of luminescence intensities at 480 nm to 530 nm can be measured to determine the magnitude of BRET, and to correct for differences in overall levels of expression of RLUC and EYFP fusion proteins. To test the feasibility of BRET ratio imaging, we collected images with a cooled-CCD camera of *E. coli* cultures expressing our constructs. Figure 4 shows images of liquid *E. coli* cultures as seen through 10 nm bandpass interference filters centered at 480 nm and 530 nm. In the cultures containing a control strain (RLUC alone) or a non-interacting combination (RLUC • KaiB & EYFP • KaiA), there is much less light emitted at 530 nm than at 480 nm. In the fusion construct (RLUC • EYFP) or the interacting combination (RLUC • KaiB & EYFP • KaiB), the amount of light emitted at 480 nm and 530 nm are roughly equal, as would be predicted from the spectra depicted in Figures 1 and 2(b2). These images can be quantified, as shown in figure 5. The 530nm/480nm ratios of luminescence for the RLUC control and the non-interacting KaiB & KaiA couple are low (about 0.4), whereas the 530nm/480nm ratios for the fusion construct and for the interacting KaiB fusion proteins are above 0.9.

#### The Use of the BRET Assay of Protein-Protein Interactions for Chemical Detection

There are several new applications using the FRET ratio principle, e.g., for measuring gene expression (Zlokarnik et al., 1998) or for measuring intracellular levels of calcium ions (Romoser et al., 1997; Miyawaki et al., 1997). BRET could be used in these applications, and may in fact have advantages over using FRET. For example, two groups have reported a novel method for ratio imaging of  $\text{Ca}^{++}$  based on using two GFP variants (e.g., BFP, CFP, GFP, YFP) linked by a  $\text{Ca}^{++}$ -binding calmodulin sequence (Romoser et al., 1997; Miyawaki et al., 1997). When the linker sequence binds  $\text{Ca}^{++}$ , it

undergoes a conformational change that brings the GFP variants closer together, enabling FRET. In the absence of sufficient  $\text{Ca}^{++}$ , the GFP variants remain far apart and FRET does not occur. Therefore, the magnitude of FRET is an indicator of  $\text{Ca}^{++}$  levels; because it is a ratio method, differences in the level of expression of the  $\text{Ca}^{++}$  indicator do not introduce artifacts to the measurement.

This principle is adaptable to BRET by designing a construct where Rluc is linked to EYfp via the same calmodulin linker. Here, the ratio of luminescence at 530 nm versus 480 nm should give an estimate of  $\text{Ca}^{++}$ . The potential advantages of the BRET approach are the same as those delineated above: a BRET indicator should be better for measuring  $\text{Ca}^{++}$  in photoresponsive or auto-fluorescent tissue, and there will be no photobleaching of fluorophores. Furthermore, because there will be no direct excitation of the acceptor fluorophore, a better ratio measurement of  $\text{Ca}^{++}$  can be obtained with BRET.

#### *Adaptability of BRET to Automation and High-Throughput Screening*

The BRET system is adaptable to means of automation and high-throughput screening.

The experiment depicted in Figures 4 and 5 suggests a relatively simple scheme for designing an in vivo library screening system for protein-protein interaction using BRET. By sensitively measuring the light emission collected through interference filters, the 530nm/480nm luminescence ratio of E. coli (or yeast) colonies expressing a "bait" protein fused to RLUC and a library of "prey" molecules fused to EYFP (or vice versa) could be measured. Colonies that express an above-background ratio could be saved and the "prey" DNA sequence further characterized. Expression vectors would need the following features: multiple cloning sites for insertion of the bait and prey libraries to enable both N-terminal and C-terminal fusions of the bait molecule and prey library to RLUC and EYFP, and an assortment of antibiotic or other markers to allow various expression/terminal fusion combinations to be tested. With appropriate instrumentation, high-throughput screening using BRET is a possibility. Using an imaging instrument similar to the one we used here, it would be possible to screen colonies of bacteria or yeast on agar plates. On the other hand, a photomultiplier-based instrument designed to measure luminescence of liquid cultures in 96-well plates could be made ideal for high-throughput BRET screening by insertion of switchable 480 or 530 nm interference filters in front of the photomultiplier.

tube.

## PREPARING THE BRET SYSTEM

### *Selecting Suitable Bioluminescent Protein-Fluorophore Pairs*

To design a system, one first chooses a bioluminescent protein with luciferase activity and a fluorophore. In nature some bioluminescent protein-fluorophore pairings interact directly with one another. In other cases the pairing interaction is mediated through other proteins, in the absence of which interactive coupling occurs with a significantly lower affinity. If such a decreased affinity is present at expression levels required to achieve BRET, when the bioluminescent protein with luciferase activity and fluorophore are coupled respectively to other protein moieties, then such a pairing is suitable for BRET. Examples of such systems which occur naturally include the *Renilla* luciferase (RLUC)/*Renilla* GFP coupling (Cormier (1978) *Methods Enzymol.* 57:237-244) and the Aequorin/*Aequorea* GFP coupling (Wilson and Brand (1998) *Annu. Rev. Cell Dev. Biol.* 14:197-230). An example of an engineered system which is suitable for BRET is an RLUC and enhanced yellow mutant of GFP (EYFP) pairing which do not directly interact to a significant degree with one another alone in the absence of mediating proteins (Y. Xu, *et al.* (1999) *PNAS* 96:151-156).

Other fluorophores which are suitable pairings for RLUC include fluorophores from GFP classes 1,2,4 and some from 5. Specific examples of suitable fluorophores include but are not necessarily limited to: wild type GFP, Cycle 3, EGFP, Emerald, Topaz, 10C Q69K, and 10C.

Other criteria which should be considered in determining suitable pairings for BRET is the relative emission/fluorescence spectrum of the fluorophore compared to that of bioluminescent protein with luciferase activity. For example it has been demonstrated that an RLUC/EGFP pairing is not as good as an RLUC/EYFP pairing based on observable emission spectral peaks (Y. Xu, (1999), *supra* and Wang, *et al.* (1997) in *Bioluminescence and Chemiluminescence: Molecular Reporting with Photons*, eds. Hastings et al. (Wiley, New York), pp. 419-422)

It should be apparent to one skilled in the art that other non-GFP fluorophores, or other bioluminescent proteins with luciferase activity may be suitable for the BRET system of the present invention if the key pairing criteria are met. Other sources for example may include pairings isolated or engineered from insects (U.S. Patent No. 5670356). Alternatively, it may be preferable even still, for the bioluminescent protein with luciferase activity and the fluorophore to be derived from completely different sources in order to minimize the potential for direct affinity between the pairings.

In a preferred embodiment a protein fusion of a blue-emitting luciferase (RLUC) and a GFP mutant acceptor fluorophore are expressed in *E. coli*. Initially, we tried EGFP as the acceptor, but as previously reported by Wang et al. (1997) with a similar fusion protein, only a barely detectable shoulder at 508 nm was added to the RLUC emission spectrum (data not shown). This shoulder was judged to be too small to use as a BRET assay for protein-protein interaction. We then tested the EYFP as an acceptor fluorophore (Orm et al., 1996), with the initial constructs used shown in figure 1(a). The RLUC●EYFP fusion construct included an intergenic sequence of 11 amino acid residues. Bioluminescence/fluorescence spectra of *E. coli* containing RLUC, EYFP, and RLUC●EYFP constructs under the control of the T7 promoter are as shown in Figure 1(b). Both the excitation and fluorescence spectra of the EYFP-expressing *E. coli* are red-shifted from EGFP, with an excitation peak at 480 nm and fluorescence peak of 527 nm. The luminescence profile of the *E. coli* cells expressing the RLUC construct displayed a typical emission spectrum for Renilla luciferase: a rather broad emission spectrum with a single peak at 480 nm (Figure 1b). However, the luminescence spectrum from cells expressing the RLUC●EYFP fusion construct yielded a bimodal spectrum, with one peak centered at 480 nm (as for RLUC), and a new peak centered at 527 nm (as for EYFP fluorescence). These data suggest that a proportion of the RLUC luminescence was transferred to EYFP and re-emitted at its characteristic wavelength.

It should be confirmed that the donor and acceptor fluorophores do not spuriously associate with each other. This can be accomplished by:

In a preferred embodiment, using RLUC and EYFP, it is confirmed that these two do not spuriously associate with each other, and thus appear to be a good BRET pair for a protein-protein interaction

assay. After co-expression of separate RLUC and EYFP (the top two constructs in Figure 1 (a)) in the same *E. coli* cells (without IPTG induction), the luminescence spectrum looks like an unaltered RLUC spectrum there is no evidence of a second peak. However, when EYFP and RLUC were overexpressed by IPTG induction, we sometimes did observe a small second peak, which suggests that there may be some weak BRET between the two proteins that can be forced by overexpression. Since we were able to achieve sufficient signal levels using the T7 promoter without IPTG addition, we could use a regime in which RLUC and EYFP do not associate by themselves. All further experiments reported in this paper were performed under conditions where we confirmed that no second peak developed in the RLUC & EYFP expressing cells.

Tests should be conducted to determine whether the donor and acceptor fluorophores still generate BRET when they are fused to interacting proteins. This can be performed by:

In a preferred embodiment, it is tested whether RLUC and EYFP would still generate BRET when they were fused to interacting proteins. As our test proteins, we chose the circadian clock proteins KaiA, KaiB, and KaiC from the cyanobacterium *Synechococcus* (Ishiura et al., 1998). Yeast two-hybrid assays indicate that KaiB proteins can form a homodimer (Iwasaki et al., 1998). Thus, we made fusion constructs of kaiB to the EYFP and RLUC genes respectively (Figure 2(a)) and measured the luminescence spectra of *E. coli* expressing these constructs. As shown in Figure 2(b2), a second peak emerges from cells expressing both RLUC•KaiB and EYFP•KaiB. The similarity between the spectrum depicted in Figure 1 (b) for the fusion construct and Figure 2(b2) strongly suggests that interaction among KaiB molecules has brought the RLUC and EYFP into close proximity such that energy transfer occurs for ~50 % of the RLUC luminescence. To demonstrate that this bimodal spectrum is specific for the KaiB interaction, we fused EYFP to KaiA and co-expressed it with RLUC•KaiB. In this case, there is no evidence of a second luminescence peak, and thus no sign of interactions between KaiA and KaiB (Figure 2(b3)). We also found no BRET-assayable interaction between KaiB and the third cyanobacterial clock protein, KaiC. This latter result is somewhat surprising because the yeast two-hybrid assay found a KaiB-KaiC interaction (Iwasaki et al., 1998). This negative result was not due to altered levels of expression or optical activity of the RLUC and EYFP fusion proteins since the RLUC signals and EYFP fluorescence levels were similar in all the *E. coli* strains. However, it may be



due to the fact that we examined only N-terminal fusions of RLUC and EYFP to the Kai proteins, and that this orientation prevented the occurrence of BRET. Whatever the explanation for the lack of KaiB KaiC interaction in our measurements, the data suggest that false positives may be rare in the BRET assay.

*Methods for Chemically or Genetically Attaching the Bioluminescent and the Fluorescent Proteins to the Proteins or Polypeptides of Interest*

There are a number of methods well known in the art for chemically or genetically attaching the bioluminescent donor protein and the fluorescent acceptor protein to the target proteins or polypeptides of interest.

## ADVANTAGES AND LIMITATIONS OF THE BRET SYSTEM

Currently, the yeast two-hybrid system is the most popular library-based technique for identifying protein interactions. Since it is a selection technique, potential interactors can be identified rapidly and further characterized without extensive screening. Other than this single (but admittedly important) advantage, optical energy transfer techniques (i.e., FRET & BRET) are potentially superior to the yeast two-hybrid system for assaying protein interactions. For example, in the yeast two-hybrid system, interaction must occur in the nucleus, but FRET or BRET interactions can be assayed in different cellular compartments by appropriate targeting of the labelled constructs. This feature is particularly important in the case of interacting membrane proteins that are unlikely to yield a positive result with the two-hybrid assay. Moreover, interactions dependent upon cell-type specific post-translational modifications that do not occur in yeast will obviously not be detected in the two-hybrid assay. Theoretically, FRET or BRET should be able to uncover these interactions. After a possible interacting pair is found with the yeast two-hybrid assay, further characterization by analytical techniques is necessary. On the other hand, FRET and BRET can be both screening and analytical techniques. Potential interactors can be identified by screening libraries in *E. coli*, yeast, or the original cell type. Subsequent FRET/BRET analyses of these candidates may then be performed in the original cell type such that the cell-type specific post-translational modification and/or compartmentalization of the proteins is preserved. Finally,

it may be possible in some cases to use FRET/BRET to assay the kinetics of changes in protein interactions *in vivo*. All of these advantages are particularly useful for investigations of protein interactions in mammalian cells, with applications to membrane protein interactions and high-throughput screening.

#### Comparison of BRET with FRET

Because BRET does not require the use of excitation illumination, it has potential advantages over FRET. BRET should be superior for cells that are either photo-responsive (e.g., retina or any photoreceptive tissue) or damaged by the wavelength of light used to excite FRET (typically near-UV; Periasamy and Day, 1998). Cells that have significant auto-fluorescence would also be better assayed by BRET than by FRET. Autofluorescence is not an uncommon problem; in addition to the cell types that have unusual autofluorescent pigments or crystals, almost all cells have broad spectrum autofluorescence of NAD(P)H elicited by near-UV light. In addition, while photobleaching of the fluorophores can be a serious limitation of FRET, it is irrelevant to BRET. A more subtle, but significant, advantage of BRET over FRET is that FRET may be prone to complications due to simultaneous excitation of both donor and acceptor fluorophores. Specifically, even with monochromatic laser excitation, it is practically impossible to excite only the donor without also exciting the acceptor fluorophore to some degree. Therefore, specific acceptor-only control experiments must be performed for FRET. In contrast, because BRET does not involve optical excitation, all the light emitted by the fluorophore must result from resonance transfer. In this respect, BRET is theoretically superior to FRET for quantifying resonance transfer., but it remains to be determined how significant this advantage will be in practical applications.

Some limitations of the BRET Assay should be kept in mind when designing the system. For example, Förster resonance energy transfer is dependent on proper orientation of the donor and acceptor dipoles. In some embodiments of the present invention, conformational states of the fusion proteins may fix the dipoles in a geometry that is unfavorable for energy transfer. Further, because the fluorophore/luciferase tags are fused to ends of the potentially interacting molecules, it is possible that some parts of the candidate molecules are interacting without allowing the fluorophore/luciferase tags to be close enough

for energy transfer to occur. Consequently, two proteins might interact in a way that is blind to the FRET/BRET technique. In other words, a negative result with a resonance transfer technique does not prove non-interaction.

Another limitation concomitant with the use of GFP variants as fluorophore tags is that GFP does not turn over rapidly. First, it takes at least an hour for the GFP molecule to fold properly (Chalfie et al., 1994; Heim et al., 1995; Heim et al., 1995). Second, once folded, GFP is remarkably resistant to proteolysis in foreign cells. Therefore, in some cases, the slow kinetics of GFP turnover may hamper measuring the kinetics of interaction. On the other hand, Renilla luciferase does not suffer these same disadvantages in turnover rate. Therefore, if one of the interacting proteins turns over more rapidly than the other, it might be useful for kinetic estimates to fuse the more labile protein to RLUC.

BRET is not without its own limitations, however. RLUC requires a substrate, coelenterazine. Coelenterazine is hydrophobic and appears to permeate many cell types (*E. coli*, yeast, plant seedlings, mammalian cell cultures), but it is possible that there are some cells that are not permeable to coelenterazine. Coelenterazine can also exhibit significant auto-luminescence in certain media. We find little if any coelenterazine auto-luminescence in simple salt media, but this may be a more serious problem in complex media. Moreover, lack of sensitivity may hinder using BRET in some cases. Depending upon the expression level of RLUC, the luminescence can be dim, and for ratio imaging only a small proportion of the total light is collected. Therefore, a very sensitive light-measuring device is necessary.

## EXAMPLES

### EXAMPLE I: CONSTRUCTION OF RLUC•EYFP FUSION EXPRESSION CASSETTE

The vectors used for creating expression cassettes of RLUC, EGFP, EYFP, and their fusions were as follows: RLUC used pRL-null (Promega, Madison, WI), while EGFP and EYFP used pEGFP-N1 and pEYFP respectively (both from CLONTECH, Palo Alto, CA). pRSET was also used as a shuttle vector (Invitrogen, Carlsbad, CA). Table 1 lists the constructs used.

Table 1. Expression strains used in this study

Strain Producing:	Plasmid	Antibiotic Resistance*
RLUC	pT7/Rluc	Km
EYFP	pT7/Eyfp	Amp
RLUC::EYFP	pT7/Rluc::Eyfp	Km
RLUC & EYFP	pT7/Rluc & pT7/Eyfp	Amp & Km
EYFP::KaiB	pT7/Eyfp:: <i>kaiB</i>	Amp
RLUC::KaiB	pT7/Rluc:: <i>kaiB</i>	Km
EYFP::KaiA	pT7/Eyfp:: <i>kaiA</i>	Amp
RLUC::KaiB & EYFP::KaiB	pT7/Rluc:: <i>kaiB</i> & pT7/Eyfp:: <i>kaiB</i>	Amp & Km
RLUC::KaiB & EYFP::KaiA	pT7/Rluc:: <i>kaiB</i> & pT7/Eyfp:: <i>kaiA</i>	Amp & Km

\*Km: Kanamycin; Amp: ampincillin

ARLUC●EGFP fusion cassette was constructed by removing the stop codon TAA of Renilla luciferase cDNA (Rluc) and adding linker sequences such that the downstream EGFP coding region was in frame with that of the upstream RLUC. Based on pRL-null DNA sequences, two primers were designed for cloning of the RLUC coding region with a T7 promoter using Pwo DNA polymerase (BOEHRINGER MANNHEIM, Indianapolis, IN): 5'-(NdeI) CATATGCTTAAGGCTAGAGTACT-3' and 5'-(ApaI) GGGCCCCGTTGTTTCATTTTGTGAGAAC-3', where the linker sequences are underlined. These primers were used to amplify a 992 bp fragment from the pRL-null template, which was then inserted into the NdeI/ApaI site of the vector pEGFP-N1 containing an enhanced green fluorescent protein gene (Egfp) to give the plasmid pRluc●Egfp under the control of a T7 promoter, yielding a P<sub>T7</sub>/Rluc●Egfp fusion expression cassette. This insertion also removed the human cytomegalovirus (CMV) promoter of pEGFP-N1. The intergenic sequence created between Rluc and Egfp was 27 bp: 5'-CGGGCCCCGGGATCCACCGGTCGCCACC-3'. This gene fusion construct was confirmed by DNA sequencing, RLUC luminescence, and GFP fluorescence.

To make the Rluc●Eyfp gene fusion, the EGFP coding region in the pRluc●Egfp was replaced with the

BamHI/NotI fragment of enhanced yellow fluorescence protein coding sequence (Eyfp) from the vector pEYFP (from CLONTECH) to produce the plasmid pT7/Rluc Eyfp, in which the EYFP open reading frame is in frame with that of RLUC. There are 11 codons between Rluc and Eyfp in this gene fusion, as shown in Figure 3(a). To create the pT7/Rluc plasmid, the EYFP coding region was removed from pRluc•Eyfp by digestion with BamHI and NotI, end-blunting and self-ligation.

## EXAMPLE II: CLONING OF CYANOBACTERIAL CLOCK PROTEIN CODING REGIONS

The recovered plasmid p48N from genetic complementation experiments that carries the cyanobacterial circadian clock gene cluster *kaiA*, *kaiB*, and *kaiC* (Ishiura *et al.*, (1998) *Science*, 281, 1519-1523), was used as a template for cloning of the *kai* coding regions. To fuse the coding region sequences for *kaiA* or *kaiB* in frame to His-Tag (a ProBond binding domain), the following two pairs of primers were designed and include the underlined linker sequences: 5'-(BglII) AGATCTATGCTCTTGGAGTATGC-3' and 5'-(HindIII) AAGCTTCGAGCTTAAGACCTCCT-3' (for cloning of *kaiA*); 5'-(BglII) AGATCTATGAGCCCTCGTAAACCTAC-3' and 5'-(HindIII) AAGCTTGCACTTAACGACAGTTAGAAG-3' (for cloning of *kaiB*), where the restriction sites are underlined and the start codons are emboldened. The 766 bp *kaiA* fragment and the 323 bp *kaiB* fragment that was amplified by Pwo DNA polymerase were each cloned into the BglII/HindIII site of the expression vector pRSET-B to give rise to plasmids pHis•*kaiA* and pHis•*kaiB*, respectively. Both the sequences of these constructs and the correct molecular weights of the fusion proteins His•KaiA and His•KaiB were confirmed by techniques known to those skilled in the art and/or as described herein.

### Fusion of the coding sequences for cyanobacterial clock proteins to Rluc or Eyfp

After the plasmid pHis•*kaiB* was digested with HindIII, the recessed 3' termini were filled by the Klenow fragment of *E. coli* DNA polymerase. The linearized plasmids were then completely digested with BamHI. The purified HindIII (blunt end)/BamHI fragments of the full length *kaiB* coding region were used to replace the EYFP coding region in the pRluc•Eyfp plasmid that had been digested with

NotI (blunt end created by Klenow fragment digestion) and BamHI to produce pRluc •kaiB, in which the kaiB coding region is in frame with that of RLUC. The construct was confirmed by restriction enzyme analysis and *in vivo* RLUC luminescence assay known to those skilled in the art and/or as described herein.

To create expression cassettes with an in-frame fusion of clock proteins to EYFP, a EYFP coding sequence without stop codon was synthesized by designing the following two primers and include the underlined linker sequences: 5'-(NdeI) CATATGGTGAGCAAGGGCGA-3' and 5'-(BglII) AGATCTCTTGTACAGCTCGTCCA-3', where the restriction sequences are underlined, and the first and last codons for EYFP are emboldened. This fragment was inserted into the NdeI/BglII sites of pHis•kaiA and pHis•kaiB respectively. This insertion removed the His-Tag and enterokinase site sequences of pRSET and produced the two plasmids pEyfp•kaiA and pEyfp•kaiB. These constructs were confirmed by DNA sequencing and *in vivo* EYFP fluorescence assay known to those skilled in the art and/or as described herein. The kaiB coding region in pEyfp•kaiB was removed by digestion with BglII and HpaI and ligated to make the plasmid pT7/Eyfp.

### **Bacterial strains and transformation**

The host strain used for cloning was *E. coli* DH5 $\alpha$ . For expression of Rluc, Eyfp, and all gene fusions that were controlled by the T7 promoter, *E. coli* BL21 (DE3) was used as a host strain (Novagen, Madison, WI). The expression host strain was transformed with single or double plasmids using competent cells prepared by calcium chloride. All transformants were confirmed by luminescence and/or fluorescence assays. Various strains containing possible combinations of fusion proteins were made and analyzed for this study. Table 1 lists those transformed strains.

### **EXAMPLE III: BIOLUMINESCENCE AND FLUORESCENCE ASSAY *IN VIVO***

*E. coli* strains were grown overnight in LB medium containing 50  $\mu$ g/ml of kanamycin and/or 100  $\mu$ g/ml of carbenicillin (for ampicillin resistant strains) at 37°C. Because LB medium has background fluorescence, the *E. coli* cultures were washed and resuspended in M9 minimal salts medium before

assay. To assay EYFP fluorescence, 1.5 ml of *E. coli* cells in M9 medium ( $OD_{600} = 0.8 - 1.0$ ) were transferred to a 2 ml cuvette. Fluorescence excitation and emission measurements were performed on a SPEX (Edison, NJ) Fluorolog spectrofluorometer with a 250w xenon arc lamp. EYFP fluorescence was excited at 470 nm and its emission scanned from 505 to 580 nm. For the luminescence assay, the lamp was shuttered off, and the cuvette was gently bubbled with air to supply sufficient oxygen for the Renilla luciferase reaction following the addition of coelenterazine (BioSynth) to a final concentration of 1  $\mu$ M. Bioluminescence emission spectra were collected between the wavelengths 440 -580 nm.

Figure 3a shows the different constructs and the linker region made for that example. EYFP was tested as an acceptor fluorophore, with the constructs shown in Fig.3a. Bioluminescence and fluorescence spectra of *E.coli* containing Rluc, EYFP and Rluc::EYFP constructs under the control of the T7 promoter are shown in Figure 3b. The luminescence profile of the *E.coli* cells expressing the Rluc construct displayed a typical emission spectrum for Rluc (Renilla luciferase) with a single peak at 480nm. (Fig.3b). The luminescence spectrum from cells expressing the Rluc::EYFP fusion construct upon coelenterazine addition, yielded a bimodal spectrum, with one peak centered at 480nm (Rluc) and a new peak centered at 527nm (as for EYFP fluorescence). These data suggest that a significant proportion of the Rluc energy was transferred and emitted at the characteristic wavelength of EYFP. Because Rluc and EYFP appear to be good BRET pair for a protein-protein interaction assay, we wanted to confirm that they do not spuriously associate with each other. After co-expression of separate Rluc and EYFP (the top two constructs in Fig. 3a) in the same *E.coli* cells (without IPTG induction), the luminescence spectrum looks like an unaltered Rluc spectrum-there is no evidence of a second peak (Fig 4b, spectrum b1).

Based on the example of interacting clock protein described in Xu et al, (1999) *Proc. Natl. Acad. Sci.* 96, p151-156), we decide to test whether the BRET assay could be used to discover interaction among the kai proteins. Thus, we made fusion constructs of kaiB to the EYFP and Rluc genes, respectively (Figure 4a). and measured the luminescence spectra of *E.coli* expressing these constructs. As shown in Figure 4b, (spectrum b2), a second peak emerges from cells expressing both Rluc::kaiB and EYFP::kaiB. The similarity between the spectrum depicted in Figure 3b for the fusion construct (Rluc::EYFP) and the Rluc::kaiB and EYFP::kaiB combination in Figure 4B (b2) strongly suggests that

interaction among kaiB molecules has brought the Rluc and EYFP into close proximity such that energy transfer between Rluc and EYFP, occurs. To demonstrate that this bimodal spectrum does not occur nonspecifically, we fused EYFP to a slightly truncated kaiA (see Figure 4a) and coexpressed it with Rluc::kaiB construct. As expected since kaiA and kaiB should not interact together, there is no second luminescence peak (Figure 4b graph b3) and thus no indication of interaction between kaiA and kaiB.

#### EXAMPLE IV: *IN VITRO* BRET ASSAY FOR KAIB-KAIB INTERACTION

In this Example, the BRET method is demonstrated by assaying interactions between proteins encoded by circadian clock genes. In cyanobacteria, *Drosophila* (Gekakis *et al.*, (1995) *Science* 270, 811-81) and mammals (King *et al.*, (1997) *Cell*, 89, 641-653; Tei *et al.*, (1997) *Nature (London)* 389, 512-516; and Albrecht *et al.*, (1997), interactions between proteins encoded by clock genes appear to play a crucial role in the function of the timekeeping loop. The circadian clock genes kai A and B from cyanobacteria (Ishiura *et al.*, (1998) *Science*, 281, 1519-1523) was therefore used to demonstrate the BRET principle.

Two expression strains, RLUC●KaiB and EYFP●KaiB, were used to examine KaiB to KaiB interaction *in vitro*, and the RLUC●EYFP fusion strain (pRluc●Eyfp) was used as a positive control. A single of colony of each strain was inoculated into LB medium with 50 µg/ml of kanamycin or 100 µg/ml of carbenicillin and grown overnight at 37°C. The cells were washed once with fresh LB medium and resuspended in the same medium containing 1 mM IPTG. After a 3 hour incubation at 37°C, the cells were collected and washed once in assay buffer (50 mM KCl, 50 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 2 mM EDTA, 5 mM DTT, 0.2% NP40, 100 µg/ml PMSF, 2 µg/ml Leupeptin, 2 µg/ml Aprotinin, and 20 mM HEPES, pH 8.0). The cells were then resuspended in fresh assay buffer at 4°C containing 10 µg/ml of lysozyme and kept on ice for 30 min. Microcentrifuge tubes containing the cells were put into a -70°C EtOH bath to quickly freeze the cells and then were placed on ice. An equal volume of fresh assay buffer was added into the cell lysates. After the mixtures were spun for 5 min in a microcentrifuge at 8,000 rpm (4°C), the supernatants were tested for fluorescence and luminescence as above except that no air was bubbled into the extracts. For monitoring KaiB-KaiB interaction *in vitro* by BRET, RLUC KaiB extracts were mixed 1:1 with EYFP KaiB extracts and thereafter incubated at room



temperature; luminescence spectra were taken at 0 min, 30 min, and 120 min after mixing.

Figure 5 represents spectra derived from mixing *E.coli* extracts from two different cultures one expressed Rluc::kaiB and the other expressing EYFP::kaiB. Immediately after mixing, there is no evidence of the second peak. After room temperature incubation for 30 minutes (second graph), a significant shoulder has emerged on the spectrum. At the 30-minutes incubation point, the intensity of the Rluc luminescence has decreased probably because of proteolysis within the extract. After 2 hours of room temperature, the luminescence exhibited a clear bimodal spectrum.

#### EXAMPLE V: BRET IMAGING OF E. COLI CULTURES

About 1.5 ml *E. coli* cultures grown overnight at 37°C in LB medium with appropriate antibiotics was washed twice with M9 medium. The cell pellet was resuspended in 300 ml of M9 medium containing 3 µM coelenterazine. Five microliters of the suspension was immediately added to the wells of a Nunc-Microwell plate. The small volume of the sample allowed good gas exchange and so bubbling was not required (fig. 6). Eight duplicates were made for each strain, which were then divided into two groups. One group was visualized through a 480 nm ( $\pm 5$  nm) interference filter, and the other through a 530 nm ( $\pm 5$  nm) filter (Ealing Electro-Optics, Holliston, MA). Images were captured with a cooled-CCD camera (TE/CCD512BKS, Princeton Instruments, Trenton, N.J.) under the control of and analyzed by custom software created by Dr. Takao Kondo (Kondo *et al.*, (1994) *Science* 266:1233-1236. This example also showed that BRET could be detected and measured using CCD-camera based technology.

Figure 7 represents the quantification analysis of luminescence intensity (in RLU) of Figure 6 when using the two interference filters 480nm and 530nm (upper graph). Lower graph shows the luminescence ratio of intensity obtained with the 530nm filter on the luminescence intensity obtained with the filter 480nm. a, b, c, and d found on both figure 6 and 7, represent Rluc, Rluc::EYFP, Rluc::kaiB and EYFP::kaiA, and Rluc::kaiB and EYFP::kaiB, respectively. The 530/480nm ratios of luminescence for the Rluc control (a) and the noninteracting kaiA and kaiB (c) couple are low (approximately 0.4), whereas the 530/480nm ratios for the Rluc::EYFP fusion construct and for the interacting kaiB fusion

proteins are above 0.9.

#### **EXAMPLE VI: DEMONSTRATION OF USE OF METHOD FOR IN-VITRO FUSION ASSAY**

Fusion assays are achieved when the Rluc moiety (donor) is attached either genetically or chemically to the fluophore acceptor moiety, the EYFP. A linker region is found in between the two moieties. Specific sites that have specific functions, could be introduced in this linker region. In this example, a specific protease site for the enterokinase was introduced between the Rluc and EYFP. Upon interaction of the enterokinase with its site in the sensor (Rluc-enterokinase-EYFP), the enzyme will cut the linker region which in turn will separate the two moieties. Therefore, we should observe a decrease of BRET (i.e. ratio 550/470nm should decrease).

The DNA constructs generated to perform this experiment are as follows: Rluc origin of the gene (pRL-CMV- Promega, Madison, WI), Rluc●EYFP origin of the gene (Xu, et al., 1999 *Proc. Natl. Acad. Sci. USA*, 96: 151-156), and Rluc:enterokinase:EYFP origin of the gene (this construct is built by introducing DNA sequences coding for the enterokinase recognition site into the linker region on the Rluc●EYFP construct).

The enterokinase site is introduced into the linker region of Rluc ●EYFP (Gly-Asp-Asp-Asp-Lys-Leu) when the enterokinase enzyme recognizes the four Asp and the lys, and cuts the peptide linker located after the amino acid Lys.

The two oligonucleotides (sense and antisense), which correspond to the enterokinase site (BRL/Gibco-Life Technologies, Gaithersburg, MD), were complementary to each other and were annealed together using molecular biology techniques well known to those skilled in the art. The double strand of DNA was engineered to have cohesive ends (BamHI (5' end) and KpnI (3' end)) after annealing and was subcloned into the Rluc●EYFP construct at the BamHI-KpnI sites.

The following are the DNA sequences corresponding to the enterokinase site (sense and antisense)

having cohesive ends:

5' GATCCGGGCGACGATGACGATAAGTTGGCGGTA3' sense oligonucleotide

5' CGCCAACTTATCGTCATCGTCGCCCCG3' antisense oligonucleotide

The three above-mentioned DNA constructs were used to produce their corresponding proteins in bacteria, which in turn were used as substrates for the enterokinase enzyme in the BRET assay (see below).

All three DNA constructs are subcloned in frame with the His tag sequences into the pQE plasmid (Qiagen, Mississauga, ON). This is done by introducing a His tag at the N-terminus of the constructs. The His tag serves to purify the different gene products from the bacterial lysats using the Nickel (Ni) beads based technology (QIAexpress kits, Qiagen, Mississauga, ON). All the constructs (Rluc gene alone, Rluc•EYFP and Rluc:enterokinase:EYFP) are subcloned into pQE-32 vector at the following restriction sites:

Table II

Construct	Construct restriction site used	Vector restriction site used (pQE-32)
Rluc	NheI*-XmaI	BamHI*-XmaI
Rluc::EYFP	NheI*-NotI*	SmaI
Rluc:enterokinase:EYFP	NheI*-NotI*	SmaI

\* all these sites were blunted before being subcloned into the pQE-32 plasmid using the Klenow enzyme under standard molecular biology techniques known to those skilled in the relevant art (Ausubel, F.M. et al., Current protocols in molecular biology, Vol. 1 (1995) John Wiley & Sons, Inc., and Sambrook, J. et al., Molecular Cloning: A laboratory manual, 2<sup>nd</sup> ed. (1989) Cold Spring Harbor Laboratory Press).

The DNA sequences for the two fusion constructs needed in this example are presented in Figures 57

and 58. Rluc DNA sequence could be found at GeneBank under the accession number : M63501

The DNA constructs in the pQE-32 vectors were then transfected into a suitable bacteria (M15) for protein expression. Bacteria was inoculated in 100ml of LB media and grown for 4 hours at 37 °C until they reached a density of 0.6 OD<sub>600</sub>. Protein expression was induced by adding IPTG (1mM) into the bacteria cultures for 16 hours at 37°C. The different proteins were purified from the bacterial lysats using 0.5ml Ni-NTA resin as described by a standard manufacturer's protocol (Qiagen, Mississauga, ON). The purified proteins were then desalted and concentrated (to a volume between 400-700ul) using a Centriprep-10 or -30 and Centricon-10 or 30 (Amicon, Beverly, MA) with a PBS + NaCl 250mM buffer. Protein concentrations were determined by the Bradford assay (Bradford, M.M., 1976, *Anal. BioChem.* 72: 248-254) using bovine serum albumin as a standard. Once purified, the proteins were used in the enterokinase BRET assay. The purity of the extracted protein, which is between 60-80%, was determined by SDS-PAGE.

The BRET assay was performed by mixing 60-100 µg of purified proteins for each well with the BRET assay buffer (PBS+ Ca2+ 1mM Mg2+ 0.5mM + glucose (1g/l) + 10mM DTT) to a total volume of 150 µl. Each assay was conducted in triplicate (i.e. three wells) in 96-well Optiplate plate (Packard, U.S.A.). Half of the assays were done in the presence of two units of the enterokinase enzyme (Invitrogen, Carlsbad, CA). Coelenterazine h (Molecular Probes, Eugene, OR) at a concentration of 20 µM/50 µl was added to each well to start the bioluminescent reaction of Rluc. The final concentration of coelenterazine h was 5 µM. The total volume of the assay was 200 µl. After the addition of the coelenterazine h, the microplates were loaded into the BRETCount™. Detection was performed using the BRETCount at 30°C for four hours. For each time point and assay, ratios were calculated by dividing the emission light output at 550nm by the emission light output at 470nm.

An example of the results are as follows. Only the ratio for the construct Rluc:enterokinase:EYFP in the presence of the enterokinase enzyme (E) decreased over time (Figure 8) demonstrating that over time more and more constructs were cut. The ratios from the negative controls (Rluc and Rluc • EYFP) did not change over time when in the presence (E) or absence of the enterokinase enzyme.

In general, this assay demonstrates that BRET can be done with partially purified proteins. Furthermore, it demonstrates the utility of the fusion type of BRET by the insertion of specific amino acid sequences (protease site, phosphorylation site or a site that recognizes analytes e.g.  $\text{Ca}^{2+}$ ) in between the two moieties.

The TopCount is a microplate scintillation and luminescence counter sold by Packard Instrument (Meriden, CT). Unlike a classical counter which utilized two photomultiplier tubes (PMTs) in coincidence to reduce noise, TopCount employs a single-PMT technology and a time-resolved pulse counting for noise reduction to allow counting in opaque standard format microplate. The use of opaque microplates can reduce optical crosstalk to negligible levels. The TopCount comes in various formats (eg. 1, 2, 6, 12 detectors (PMTs)), which could read 1, 2, 6 or 12 samples simultaneously depending on the format.

A modified 2-detector TopCount (BRETCount) was used to measure BRET. Modifications included positioning the bandpass interference filters under each TopCount PMT (between the PMT and the detector mounting plate), and adjusting the TopCount software to allow each PMT to read each well of a microplate. These changes allow the sequential detection of light at specific wavelengths emitting from the samples. Accordingly, for each well (or sample) we obtained two light output values corresponding to each filter. Beside its throughput (microplate reader), the TopCount is a temperature controlled instrument that is useful when performing cell-based assays or timecourse experiments.

The bandpass interference filters were designed based on emission spectra found in Figure 3b and 22. The two filters used were 1) for the acceptor emission (fluorophore): 550DF80 (Omega Optical Inc, Brattleboro VT); and 2) for the donor emission (bioluminescent protein): 470DF60 (Omega Optical Inc, Brattleboro VT).

BRET is measured by either dividing the light output for the 550 nm filters by the 470nm (acceptor/donor) or the opposite. The instrument parameters used for the BRETCount were luminescence SPC mode and read length of 1 sec for each PMT.

**EXAMPLE VII: CONSTITUTIVE PROTEIN-PROTEIN INTERACTION**

The interaction of the kaiB domain, which is involved in the circadian cycle of bacteria, with itself has been well documented (Xu, et al., 1999, Proc. Natl. Sci. USA, 96:151-156). This system is composed of three proteins identified as kai A, B, C, and is used to assay the BRET technology.

The kai based constructs (Xu, et al., 1999, Proc. Natl. Sci. USA, 96:151-156) were subcloned into mammalian expression vectors as follows: The two constructs (pT7/EYFP-kaiB; pT7/EYFP-kaiA) were cut with the restriction enzyme XbaI-HindIII to retrieve the resulting fusion gene (EYFP-kaiB; EYFP-kaiA). The remaining construct pT7/Rluc-kaiB was cut with the restriction enzyme NheI-HpaI to retrieve the Rluc-kaiB fusion gene. The fusion genes were then inserted into the multiple cloning site of pCDNA3.1 (-) zeo (Invitrogen, Carlsbad, CA) and digested with the same enzyme used to create the respective fusion gene.

The BRET assay was performed as follows. First, CHO-K1 cells were transiently transfected using the kit LipofectAMINE™ (BRL/Gibco-Life technology, Gaithersburg, MD) in 100mm dishes with the following combinations of the DNA constructs: pCDNA3.1/Rluc-kaiB alone; pCDNA3.1/Rluc-kaiB + pCDNA3.1/EYFP-kaiB, and pCDNA3.1/Rluc-kaiB + pCDNA3.1/EYFP-kaiA.

When combinations of vectors were used, a total amount of 8 µg of DNA was used. Each cell sample was transfected with 4 or 0.5 µg of each construct with enough carrier DNA (pCDNA3.1 (-) wild type) to bring the amount up to a total of 8 µg.

Following transfection, the cells were washed 48 hours later with a mixture of PBS Ca<sup>2+</sup> 1mM/ Mg<sup>2+</sup> 0.5mM + glucose 1g/l + aprotinin 2 µg/ml +DTT 10mM and counted. The transfected cells are distributed into a 96-well white Optiplate (Packard, U.S.A.) at a sufficient density to ensure a good bioluminescence reaction (in this example, 50 000 cells per well in 150 µl were used). The cells were plated into the same buffer that was used for the wash. To start the bioluminescence reaction, 50 µl of coelenterazine h (Molecular Probes, Eugene, OR) at a concentration of 20 µM was added to each well.

The final concentration of coelenterazine was 5 $\mu$ M. The microplates were then loaded into the BRETCount for the light emission measurements at 25°C using a read length of 1 sec per filter.

### EXAMPLE VIII: REGULATED PROTEIN-PROTEIN INTERACTION

The cAMP response element binding (CREB) protein is a well known transcription factor involved in the cAMP pathway. When cellular cAMP levels increase by activation of the cAMP pathway, it triggers the activation of the protein kinase A (PKA) which in turns phosphorylates CREB. Once phosphorylated, CREB is recognized by other cellular factors like the CREB binding protein (CBP). The CBP then binds to CREB using a specific protein domain. Phosphorylated CREB alone or bound to CBP stimulates transcription activities of promoters that have a cAMP response element (CRE).

In the following example, we used two methods to increase the cellular cAMP levels:

First, a chemical method using forskolin that activates directly the enzyme that produces cAMP, the adenylate cyclase is used. Second, cAMP levels may be increased biologically using a G-protein coupled receptor (GPCR) and an agonist for the receptor. Examples of GPCRs that may be used include the calcitonin receptor and its agonist calcitonin, and the b2 receptor (adrenergic receptor) with its agonist isoproterenol. Both receptors are know to be coupled to a Gs G-protein which stimulates cAMP production. We also used a receptor (CCR5) that is coupled to a Gi protein which decreases cAMP levels.

The CREB domain needed for the experiment was found between amino acid 1-283. This region is well known to be recognized and phosphorylated by PKA on the ser133. This region has been amplified by PCR using the vector pFA-CREB (Stratagene, La Jolla, CA) as a template, which encodes the full length human CREB gene. The primers used for the PCR reaction are as follows:

oligonucleotides sense: 5'GGAAGATCTATGACCATGGACTCTGGAG; and  
oligonucleotide antisense: 5' CCAAGCTTTGCTGCTTCTTCAGCAGG.

To facilitate the subcloning, the oligonucleotides were engineered with two different restriction sites (one per oligonucleotide) at their 5' end. The sense oligonucleotide has a Bgl II restriction site and the antisense oligonucleotide has a Hind III restriction site. Hence, after the amplification, the amplified product was purified and digested with these enzymes. The pT7/EYFP-kaiB (Xu, et al., 1999 *Proc. Natl. Acad. Sci. USA*, 96: 151-156) was digested using the same restriction enzyme to remove the kai B gene. The amplified CREB domain was then subcloned at the C-terminal of EYFP in the pT7/EYFP construct. A linker of two amino acid was created between the EYFP and the CREB domains.

The novel fusion protein (EYFP-CREB) from the bacterial pT7 expression vector, was then subcloned into the mammalian expression vector pCDNA3.1(+) zeo (Invitrogen, Carlsbad, CA). For this purpose, the pT7 EYFP-CREB was digested with XbaI and HindIII and the pCDNA3.1 was digested by NheI (which is XbaI compatible) and HindIII.

A SV40 nuclear localization signal (NLS) was introduced at the C-terminus of the fusion EYFP-CREB gene in order to ensure that the construct once expressed, was localized in the nucleus. We anneal two new complementary oligonucleotides encoding the following SV40 NLS: Pro-Lys-Lys-Lys-Arg-Lys-Val-stop codon:

Oligonucleotide sense: 5' GATCCCCCAAGAAGAAGAGGAAGGTGTGACCGGTC

Oligonucleotide antisense: 5' TCGAGACCGGTACACCTTCCTCTTCTTCTTGGGG

Once the oligonucleotides were annealed, they form a double strand DNA having two cohesive restriction sites at both ends (BamHI at the 5' and AgeI at the 3'). The construct pCDNA3.1-EYFP-CREB was digested using these enzymes and the annealed double oligonucleotide was subcloned. A linker region of eight amino acids was created between the CREB and the NLS. The final construct is referred to as pCDNA3.1/EYFP-CREB-NLS.

The CBP domain needed for the experiment was found between amino acid (462-661). This region is well known to bind phosphorylated CREB (Chrivia, J.C., et al., 1993, *Nature*, 365:855-859; Shih, H-M, et al., 1996, *Proc. Natl. Acad. Sci. USA*, 93:13896-13901; Kwok, R.P.S., et al., 1994, *Nature*,



370: 223-226; Arias, J., et al., 1994, Nature, 370.:226-229). This region has been amplified by RT-PCR using a cDNA library of human placenta. This library was made using mRNA from human placenta (from Clontech, Palo Alto, CA) and the Marathon ready kit (Clontech, Palo Alto, CA). The oligonucleotides used are as follows:

oligonucleotide sense 5' CGGGTACCGACAGGGCAACAGAATGCC

oligonucleotide antisense 5' ATGCGGCCGCTATCTTGTAGATTTTCTCTGC

To facilitate the subcloning, the oligonucleotides were engineered with two different restriction sites (one per oligonucleotide) at their 5' end. The sense oligonucleotide has a Kpn I restriction site and the antisense oligonucleotide has a Not I restriction site. After the amplification, the amplified product was purified and digested with these enzymes. The pCDNA3.1/Rluc-EYFP (made at BioSignal from the pT7/Rluc-EYFP vector) was digested using the same restriction enzyme to remove the EYFP gene. The amplified CBP domain was then subcloned at the C-terminal of Rluc into the pT7/Rluc-EYFP construct lacking the EYFP gene. A linker of eight amino acid was created between the EYFP and the CREB domain.

A SV40 nuclear localization signal (NLS) was introduced at the C-terminus of the fusion Rluc-CBP gene in order to ensure that the construct once expressed, was localized in the nucleus. Two new complementary oligonucleotides were annealed that encoded the following SV40 NLS: Pro-Lys-Lys-Lys-Arg-Lys-Val-stop codon:

oligonucleotide sense corresponding to the NLS: 5'

TCGAGGATTCCCAAGAAGAAGAGGAAGGTGTAGGTTT

oligonucleotide antisense corresponding the NLS.: 5'

AAACCTACACCTTCCTCTTCTTCTTGGGGAATTC

Once annealed, the oligonucleotides form a double strand DNA having one cohesive restriction site at the 5' end XhoI and a blunt end at the 3'. The pCDNA3.1-Rluc-CBP was digested using XhoI

enzyme and PmeI (generating a blunt end). The annealed double oligonucleotide was subcloned into these sites. A linker region of six amino acids was created between the CBP and the NLS. The final construct is referred to as pCDNA3.1/Rluc-CBP-NLS.

DNA sequences for all the constructs needed in this example are presented in Figures 60 and 61.

#### **BRET assay:**

The pCDNA3/Rluc-CBP-NLS and pCDNA3.1/EYFP-CREB-NLS DNA constructs were transiently transfected into CHO-K1, CHO-CCR5 or CHW- $\beta$ 2 cell lines using LipofectAMINE™ (BRL/Gibco-Life technology, Gaithersburg, MD) using equal amount (4  $\mu$ g) of DNA for each construct in 100mm dishes.

**BRET assay using the transfected cells in suspension.** At 48 hours post-transfection, the transfected cells were starved for 2 hours in a mixture of PBS + Ca<sup>2+</sup> 1mM /Mg<sup>2+</sup> 0.5mM + glucose 1 g/l + aprotinin 2  $\mu$ g/ml at 37°C before being harvested, washed with a mixture of PBS Ca<sup>2+</sup>/Mg<sup>2+</sup> + glucose + aprotinin 2  $\mu$ g/ml and counted. For the BRET assay, to ensure a sufficient signal, 50 000 cells per well was used in 96-well white Optiplates (Packard, Meriden, CT).

**BRET assay using transfected cells attached onto the bottom of the microplate.** In some experiments, cells were harvested at 24h post-transfection and seeded onto a 96-well white CulturePlate™ microplates (Packard, Meriden, CT) at a density of 25 000 cells per well. At 48 hours post-transfection, the transfected cells were starved as previously mentioned, washed and then analyzed without being harvested.

The assay conditions for both methods (cell in suspension or attached), cells were in PBS Ca<sup>2+</sup> + Mg<sup>2+</sup> + glucose + aprotinin 2  $\mu$ g/ml in volume of 100 $\mu$ l. Ligands or forskolin in PBS Ca<sup>2+</sup> + Mg<sup>2+</sup> + glucose (at various concentrations see graphs) was added to the cells to make an intermediate volume of 150  $\mu$ l. To this, 50  $\mu$ l of coelenterazine h (20mM in PBS Ca<sup>2+</sup> + Mg<sup>2+</sup> + glucose + aprotinin 2  $\mu$ g/ml) was added in each well to start the bioluminescent reaction (final concentration of coelenterazine h 5  $\mu$ M).

Total volume of the reaction 200  $\mu$ l. After the addition of coelenterazine h in each well, the microplate was loaded into the BRETCount for detection at 25°C. Each well was read using both filters for 1 second each.

#### **Effect of adenylate cyclase activation by forskolin or calcitonin receptor on cAMP sensor**

The CHO-K1 cells were transfected with both constructs. At 48 hours post-transfection, the cells were starved for 2 hours, washed, harvested and counted. A total of 50 000 cells was added to a 96-well microplate. Forskolin was added to each well to a final concentration of 10  $\mu$ M. Coelenterazine h was then added in each well to start the reaction. The microplate was loaded into the BRETCount and light outputs at 470nm and 550nm were determined at several points to determine a timecourse (fig. 10). As for forskolin, the agonist calcitonin was also used to increase cAMP levels. The CHO-K1 cells express the calcitonin receptor endogenously, and once activated, couples to the Gs type G-protein. The Gs type G-proteins are known to activate adenylate cyclase and hence increase cellular cAMP levels. In this example, 2 nM and 10nM of calcitonin were used. The results demonstrate the sensor is sensitive to changes of cAMP level. Ratios increased over time in presence of calcitonin or forskolin compared to wells that have not been induced (buffer alone). Each time point represents mean  $\pm$  SD of four wells (assays are done in quadruplicate).

The timecourse experiment of Figure 10 was repeated using attached cells this time and various concentration of calcitonin (ranging from 0 to  $3.1 \times 10^{-8}$  M). Dose response curve (Figure 11) was built using the data point at 45 minutes after addition of coelenterazine. The dose response determined an EC50 of 0.027 nM which was close to those found in literature (0.03-0.06 nM) for a functional assay. Each time point represents mean  $\pm$  SD of four wells (assays are done in quadruplicate).

#### **Effect of the activation of an overexpressed GPCR on the cAMP sensor**

Instead of using an endogenous GPCR or forskolin to increase cellular cAMP levels, the constructs were transfected into a stable cell line expressing an heterologous GPCR. The CHW-  $\beta$ 2 cell line was used for this purpose. It expresses the human adrenergic  $\beta$ 2 GPCR (level of expression: 0.77 pmole/mg

of membrane protein) and like calcitonin receptor it is coupled to Gs G-protein. It had been shown that this receptor is functionally coupled to the cAMP pathway; upon binding of its agonist the isoproterenol, it triggers the production of cellular cAMP. At 48 hours post-transfection of the sensor constructs, the cells were starved, washed, harvested and counted as mentioned above. A total of 50,000 transfected cells per well was added into a 96-well white Optiplate (Packard, Meriden, CT) along with two concentrations of isoproterenol. In several wells, the cells were incubated with forskolin as control (as above). Figure 12 represents the induction of  $\beta 2$  receptors by isoproterenol over time. Again, the ratios increased in the presence of isoproterenol or forskolin, indicating that the two moieties (Rluc and EYFP) are brought in close proximity when cAMP levels increase.

Figure 13 represent the dose response of isoproterenol in the transfected CHW- $\beta 2$  cells from another experiment. In this case, the transfected cells were analysed at 24 hours post-transfection. EC50 of 0.013 nM was calculated from the curve. This value is similar to values found in literature for  $\beta 2$  receptor using a functional assay known to those skilled in the art.

### **Gi coupled receptor**

The cAMP sensor could be used to monitor receptor activation (upon ligand binding) for the Gs-coupled receptor since the Gs-coupled receptors are known to increase cellular cAMP levels. This experiment examined if the cAMP sensor be used for Gi-coupled receptor. These receptors couple to different G-proteins that decrease cellular cAMP levels once the ligand binds to its receptors. A stable cell line that constitutively expressed the chemokine CCR5 receptor was used (BioSignal, Montreal, PQ). The CCR5 is coupled to a Gi type G-protein once it is activated. Two different approaches were used to determine if the cAMP sensor could monitor the decreased cAMP levels.

The first method used transfected CHO-CCR5 cells plated in 100mm dishes. These cells were starved and seeded in 96-well plates as previously described. They were then induced with forskolin at a final concentration of 1  $\mu$ M to increase their cAMP content and with MIP-1 $\beta$ , which is a selective agonist for the CCR5 receptor. Coelenterazine was added in each well to start the reaction.

Alternatively, the cells were not starved and only MIP-1  $\beta$  was added. Since cAMP levels are naturally high in cells we did not starve the cells to "artificially" increase cAMP level with forskolin in order to detect the inhibitory effect of the CCR5 on the adenylate cyclase.

Figures 14 and 15 represent the above two approaches, respectively. In both cases, the activation of the CCR5 by its agonist was monitored. Ratios decrease over time in the presence of the agonist MIP-1  $\beta$ .

#### **EXAMPLE IX: PROTEIN-PROTEIN INTERACTION USING TYROSINE KINASE RECEPTOR (TKR)**

This assay /sensor is similar to the CBP:CREB assay. It detects TKR activities (Heldin, C.H., et al., 1998, *Biochim. Biophys. Acta*, 1378: F79-F113). The TKRs are a family of receptors that share one transmembrane region (instead of 7 like the GPCRs) and have tyrosine kinase (TK) activities. Examples of this family include EGF, PDGF, insulin, etc. The TK domain is localized at the C-terminus of the protein inside the cell. This domain is regulated by the ligand binding domain localized at the N-terminus (outside the cell). Upon ligand binding, the receptor homo-dimerizes and the TK domain is activated. Once activated, one receptor will phosphorylate (on a tyr) its partner (autophosphorylation). This phosphorylation is then recognized by cellular adapter proteins which in turn activates downstream effectors. Two of these adaptor proteins were engineered: SHC and Grb2. Both of these proteins could recognize and bind to a tyr phosphorylated receptor (at different sequences). Most often (at least for the EGF and PDGF receptors), these proteins act sequentially (Yajnik, V., et al., 1996, *J. Biol. Chem.*, 271: 1813-1816; Sasaoka, T., et al., 1996, *J. Biol. Chem.*, 271:20082-20087; Gram, H., et al., 1997, *Eur. J. Biochem.*, 246:633-637; Rozakis-Adcock, M., et al., 1992, *Nature*, 360: 689-692; Sakaguchi, K., et al., 1998, *Mol. Endocrin.*, 12: 536-543). The adaptor protein SHC first recognized and bound to the tyr phosphorylated receptor (directly at the specific phosphorylation site). The protein domain responsible for the binding of SHC to the receptor is found at the N-terminus of SHC and it is called phosphotyrosine binding (hereafter PTB). This domain has a different sequence signature compared to the well known prototype SH2 which shares the same phosphorylated tyr binding activity (Yajnik, V., et al., 1996, *J. Biol. Chem.*, 271: 1813-1816; Schaffhausen, B., 1995, *Biochem. Biophys.*

Acta, 1242: 61-75; Songyang, Z., et al., 1993, Cell, 72: 767-778). Once bound to the receptor, SHC is then phosphorylated (at a specific tyr) by the receptor. The Grb2 then recognizes and binds the phosphorylated SHC using its SH2 domain (SH2 of Grb2:SHC has also a SH2 domain but it is used for other signal pathway) (SH2: Src homology 2). In our assay, we measured this link between SHC and Grb2 in order to monitor the RTK activity.

In order to build the assay, it was necessary to fuse the protein domains involved in the RTK activity, with the Rluc and the EYFP. The following is a description of the procedure.

### EYFP-SH2

SH2 was fused at the C-terminus of EYFP. SH2 domain of Grb2 (amino acid 49-164) was amplified by PCR (standard technique) using as template an human EST (expressed sequence tag) and specific oligonucleotides:

Oligonucleotide sense : 5' GGAAAGATCTCCCAAGAACTACATAGAAATG

Oligonucleotide antisense: 5' CCAAAGCTTTCAGAGGGCCTGGACGTATG

EST No: human Image clone no. 26670

To facilitate the subcloning, the PCR oligonucleotides were engineered with two different restriction sites (one per oligonucleotide) at their 5' end (underlined). Sense oligonucleotide has a Bgl II and antisense oligonucleotide has a Hind III site. Hence, after the amplification, the amplified product was purified and digested with those enzymes. The pT7/EYFP-kaiB (Xu, ) was digested using the same restriction enzyme to remove kai B gene. The amplified CREB domain was then subcloned at the C-terminal of EYFP in the pT7/EYFP. A linker of 2 amino acid was created between the EYFP and the CREB domain.

The EYFP-SH2 fusion gene was then subcloned into the pCDNA3.1 (-) hygromycin mammalian expression vector using the restriction sites XbaI and HindIII.

### **Rluc-PTB**

PTB domain of SHC was fused to the C-terminus of Rluc. PTB domain of SHC (amino acid 111-487 of the p66<sup>SHC</sup> variant) was amplified by RT-PCR using as template a HeLa cDNA library. This library was made using total RNA from HeLa cell and the SuperScript II Rnase H<sup>-</sup> Reverse Transcriptase (BRL/Gibco-Life Technologies, Gaithersburg, MD) with the oligo dT primer as described in manufacturer protocol.

PCR was performed using standard technique known to those skilled in the art and the following primers:

Oligonucleotide sense 5' CGGGTACCGATGAACAAGCTGAGTGA

Oligonucleotide antisense: 5' TAGCGGCCGCTCAGGGCTCCCCTCGGAGC

To facilitate the subcloning, the PCR oligonucleotides were engineered with two different restriction sites (one per oligonucleotide) at their 5' end. Sense oligonucleotide has a KpnI and antisense oligonucleotide has a NotI site. Hence, after the amplification, the amplified product was purified and digested with those enzymes. The pCDNA3.1/Rluc-EYFP (this construct was made at BioSignal (Montreal, Canada) using the pT7/Rluc-EYFP from Vanderbilt University using the restriction sites NheI-NotI of pCDNA3.1 zeo (+)) was digested using the same restriction enzyme to remove EYFP gene. The amplified PTB domain was then subcloned at the C-terminal of Rluc in the pT7/Rluc. A linker of 8 amino acid was created between the Rluc and the PTB domain.

DNA sequences for the two constructs needed in this example are presented in Figures 62 and 63.

### **BRET assay**

The two constructs pCDNA3.1/Rluc-PTB and pCDNA3.1/EYFP-SH2 were co-transfected into GH4-C1 cell line with LipofectAMINE (BRL/Gibco-Life Technologies, Gaithersburg, MD) using manufacturer protocol in 100mm dishes. The GH4-C1 cell line was used because it is known that these cells express endogenously the EGF receptors needed for the assay. At 48 hours post transfection, transfected cells were starved in MEM without serum for 2 hours, washed with PBS Ca<sup>2+</sup>/Mg<sup>2+</sup> + glucose + aprotinin, harvested and seeded in 96-well microplate at a cell density of 50,000 cell per well in the PBS Ca<sup>2+</sup>/Mg<sup>2+</sup> + glucose + aprotinin assay buffer. The agonist EGF was then added at various concentration to make at this point a total volume of 150ml. 50ml of coelenterazine h (at a concentration of 20mM) was added in each well to start the bioluminescent reaction. Microplates were loaded in the BRETCount for the measurement of Rluc and EYFP emissions. Read time for each filter: 1sec. Temperature of detection was at 25 °C.

Figure 16 represents a dose response of EGF on the transfected cells at 45 minutes post induction (with EGF). From this curve, a EC<sub>50</sub> of 0.92nM was calculated which is similar of the value found in literature. This sensor is a valuable tool to monitor TKR activity and hence would be useful for drug discovery.

#### **EXAMPLE X: PROTEIN-PROTEIN INTERACTION USING TOPAZ INSTEAD OF EYFP**

Evidence that GPCR associate to form dimer or multimeric species remain challenging and rely solely on biochemical experiments performed *in vitro*. In this example, we set to investigate if GPCR dimerization occurs in live cell. For that purpose, the moieties were fused in frame at the C-terminus of b2 receptor. Hence two fusion constructs were generated: 1- b2-Rluc and b2-Topaz. Topaz is another GFP variant having similar spectral proprieties when compared to EYFP.

The following assay demonstrates the results showing  $\beta$ 2 dimerization in live cells using BRET.

The DNA of both Rluc (from pRL-CMV, Promega) and Topaz (pGFPTpz-N1, Packard) genes were inserted at the C-terminus of the human  $\beta$ 2 gene without its stop codon, having spacers of four and five amino acids, respectively. Topaz is used instead of EYFP because it has a higher extinction coefficient



and quantum yield. Both fusion proteins were then subcloned into a pRSV vector to permit their expression in mammalian cells.

HEK-293 and CHO-K1 cells were transiently transfected using  $\text{Ca}^{2+}$ -phosphate precipitation and LipofectAMINE™ (BRL/Gibco, Gaithersburg, MD) respectively in 100mm dishes. Transfection efficiencies between 40-60% were generally obtained using this technique (when analyzed using a GFP reporter vector and fluorescence microscope) for CHO-K1 cells and about 20-30% for HEK-293 cells. The cells were always transfected with the same amount of total DNA.

The assays were performed 48 hours post transfection in quadruplicate. The cells were harvested using versene (PBS+EDTA), counted and diluted in PBS (containing  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ) to about 50,000 cells per well in a white Optiplate (Packard, Meriden, CT). The assays may be performed using either suspension or adherent cells. When the assays were performed with adherent cells, the cells were harvested from 100mm dishes 24h post-transfection, counted and seeded into a 96-well plate before the detection step performed the following day. Light emission was triggered by the addition of coelenterazine 5  $\mu\text{M}$ . Emissions were detected at various times after addition of the coelenterazine using the BRETCount. Parameters used for the BRETCount were luminescence SPC mode, a read length of 1 sec for each wavelength at 25°C, and 470DF60 550DF80 filters.

#### **1- Pharmacological characterization of the fusion $\beta 2$ -Rluc and -Topaz proteins.**

For the pharmacological characterization, 293 cells were transfected with either a  $\beta 2$ -wt expressing vector,  $\beta 2$ -Rluc expressing vector or  $\beta 2$ -Topaz expressing vector. Saturation binding experiments using isoproterenol were performed on the transfected 293 cells. Table III shows the  $K_D$  obtained from the saturation curves. Values obtained for the fusion proteins were significantly different from  $\beta 2$  wt receptor but fusion proteins still show a high affinity for the agonist.

Table III:  $K_D$  generated from the transient transfected 293 cells (pM).

	<i><math>\beta</math>2-wt</i>	<i><math>\beta</math>2-Rluc</i>	<i><math>\beta</math>2-Topaz</i>
K <sub>D</sub>	37	59	113

## 2- Detection of $\beta$ 2 receptor dimerization using BRET.

For the detection of  $\beta$ 2 receptor dimerization, 293 cells were transfected with  $\beta$ 2-Rluc vector alone or in combination with  $\beta$ 2-Topaz or CCR5-Topaz. The results of this assay (Figure 17), also included the results of tri-transfections where  $\beta$ 2-Rluc and  $\beta$ 2-Topaz transfected cells were further transfected with  $\beta$ 2 wt or V2 receptor DNA. At 48h post-transfection, the cells were harvested, washed, counted and distributed into a 96-well plate (50 000 cells per well) in PBS. After the addition of coelenterazine, microplates were read at different times (Figure 17). In Figure 18, the cells were harvested at 24h post-transfection, counted and distributed into 96-well plates before the analysis at 48h. By using this procedure, the cells adhere to the bottom of the well before the analysis. Although this process does not alter the cells, the ratios obtained tend to be lower compared to analysis performed with harvested cells.

Figures 17 and 18 demonstrate that the BRET ratio is increased when  $\beta$ 2-Rluc is co-expressed with  $\beta$ 2-Topaz compared to  $\beta$ 2-Rluc alone, indicating that these fusion receptors were in close proximity (dimerized). Hence, even in absence of agonist, part of the receptor pool is found in a dimerized form. This association was stable for at least 20 minutes (Figure 17) even as the Rluc/coelenterazine reaction continues. This association could be perturbed by the addition of  $\beta$ 2 wt DNA construct (at 5 times the amount of the fusions) during transfection (diamond, Figure 17). In contrast, V2 receptor expression had no effect on the dimerization of  $\beta$ 2 receptors. CCR5 receptor was fused to Topaz as for  $\beta$ 2, and used as negative control (Figure 18). This fusion protein was characterized pharmacologically (data not shown). When co-transfected with  $\beta$ 2-Rluc, a significant increase of the BRET ratio was observed.

## 3- Kinetics of $\beta$ 2 receptors dimerization

The effect of the agonist isoproterenol on the dimerization state of  $\beta 2$  receptors was determined. After harvesting the cells, cells were pre-incubated for 10 minutes with 1 mM of isoproterenol at 37 °C. After this period of time, the cells were distributed into the 96-well plate and coelenterazine was added to start the reaction. BRET analysis was then proceeded at 25 °C in the BRETCount. Figure 19 shows that BRET ratio increased for 15 minutes and then started to decline.

#### **4- Dose-response experiments.**

Dose response experiments on the transiently transfected cells was also performed. At 48 hours post transfection, the cells were harvested, seeded into 96-well plate and incubated with various concentration of isoproterenol. After 10 minutes at 37 °C, coelenterazine was added to start the Rluc reaction. The addition of the agonist generated a dose-response curve with a EC<sub>50</sub> of 2nM (Figure 20). This EC<sub>50</sub> value obtained by BRET analysis is similar to the one obtained in ligand binding analysis. Similar results were obtained when assays were repeated using adherent cells instead of suspended cells.

The use of the BRET technology in the above examples demonstrates for the first time  $\beta 2$  receptor dimerization in live cells. Previously,  $\beta 2$  dimerization had only been shown by immunoprecipitation experiments. The use of the BRET technology also showed that the addition of agonist affects dimerization by the modification of the BRET ratio. BRET assays using other agonists, antagonists and even inverse agonists (data not shown) were also performed and have generated pharmacological data. The above examples reflective of the application of the BRET technology.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Consequently, such changes and modifications are properly, equitably, and "intended" to be, within the full range of equivalence of the claims to follow.

**EXAMPLE XI: DEMONSTRATION THAT THE METHOD IS AMENABLE TO AUTOMATION.**

To test the feasibility of BRET ratio imaging, images were collected with a cooled-CCD camera of *E. coli* cultures expressing certain constructs. For these applications, the ratio of luminescence intensities at 480 nm to 530 nm can be measured to determine the magnitude of BRET, and to correct for differences in overall levels of expression of RLUC and EYFP fusion proteins.. Figure 4 shows images of liquid *E. coli* cultures as seen through 10 nm bandpass interference filters centered at 480 nm and 530 nm. In the cultures containing a control strain (RLUC alone) or a non-interacting combination (RLUC•KaiB & EYFP•KaiA), there is much less light emitted at 530 nm than at 480 nm. In the fusion construct (RLUC•EYFP) or the interacting combination (RLUC•KaiB & EYFP•KaiB), the amount of light emitted at 480 nm and 530 nm are roughly equal, as would be predicted from the spectra depicted in Figures 1 and 2(b2). These images can be quantified, as shown in figure 5. The 530nm/480nm ratios of luminescence for the RLUC control and the non-interacting KaiB & KaiA couple are low (about 0.4), whereas the 530nm/480nm ratios for the fusion construct and for the interacting KaiB fusion proteins are above 0.9.

Because of the better throughput of the BRETCount compared to the LumiCount, it is possible to develop and test various assay parameters to improve the detection of the BRET signal and to adapt BRET detection for high throughput screening (HTS). The assay parameters were characterized. Parameters tested were: cell number per well: type of media: the use of protease inhibitor: effect of the pH: effect of HEPES: effect of BSA: effect of DMSO: other.

CHO-K1 cells were transiently transfected using LipofectAMINE (BRL/Gibco Life Sciences, Gaithersburg, MD) in 100mm dishes according to the manufacturer's protocol (0.2mg DNA per construct + X mg of carrier DNA for a total of 8 mg). Transfection efficiencies between 40-60% using this technique were obtained (when analyzed using a GFP reporter vector and fluorescence microscope). 48 hours post transfection, cells are harvested using a low concentration of trypsin, counted and diluted in D-PBS (containing  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ). For most of the examples given below, 50,000 cells per well were used in white Optiplate (Packard, Meriden, CT). The assays were performed in

quadruplicate in a total of 200  $\mu$ l. Light emissions were triggered by the addition of coelenterazine h (5  $\mu$ M; addition of 50  $\mu$ l of a stock 20  $\mu$ M).

The assays were conducted using the BRETCount at various times after the addition of the coelenterazine. Parameters used for the BRETCount were: luminescence SPC mode, a read length of 1 sec for each wavelength at 25°C, 470DF60 and 550DF80 filters.

Spectral analysis of BRET reaction were performed using a Spex instrument (Instrument S.A.) with the excitation lamp turned off, an integration time of 0.4sec, Scan 400-600nm, emission slit 10-12nm, step 2nm, at 25°C (fig. 22).

To follow are examples of assays demonstrating the effect of changing different assay parameters:

#### **1- Cell number per well.**

A standard BRET assay was performed (Figure 21) using the BRETCount with the assay parameters used for the LumiCount (modified LucLite buffer, coelenterazine h 5  $\mu$ M, white Optiplate, total volume of 200  $\mu$ l, etc).

BRET ratios were very stable over time. The positive control slightly increased and at the same time both negative controls slightly decreased. Rluc emission and energy transfer peaks change their shape during the light emission (see Figure 22). The ratio increased over time because the Rluc/coelenterazine reaction decays producing less light but the energy transfer is still at its maximum efficiency. The effect of cell number per well is shown in Figure 23. Different total cell numbers were distributed into wells of the microplate. After the addition of the coelenterazine h, the microplate was read at different times. Figure 23 shows that number of cells per well has little effect on the ratios. As little as 2000 cells per well (in a 96-well plate) were used without significant changes in the ratios. Note that at this low number of cells, observed large variations in the BRET ratios late (over 75min.) in the assays were observed, which may be attributed to the numerous plate movements and cell adhesion to the vessel.

## 2.- Type of media.

The effect of the type of media on the luminescence of the Rluc was assayed in absence or in the presence of various amount of DTT. Transfected CHO cells were collected and redistributed in 96 well plate using different media conditions (Figures 24 - 26). Luminescence timecourses at 470nm (TopCount) were performed at 25 °C upon coelenterazine addition. The effects of three different media were assayed: PBS  $\text{Ca}^{2+}/\text{Mg}^{2+}$  (Figure 24), PBS  $\text{Ca}^{2+}/\text{Mg}^{2+}$  + glucose (Figure 25) and DMEM without phenol red (Figure 26).

In absence of DTT, PBS alone gave higher Rluc long term emission and hence the best long term Rluc emission stability, followed by PBS+glucose and DMEM. Similar results were found in presence of 100mM DTT: PBS alone gave higher long term counts. As expected, increasing the concentration of DTT improved Rluc stability in all three media. Unfortunately, high amounts of DTT (50 and 100mM) which gave the best stability, damaged the cells: viability was assayed using trypan blue dye and decreased more than 20% over a period of 2 hours. A good compromise between viability (less than 15%) and Rluc stability was achieved at 10mM. This concentration was chosen for the BRET assays. The addition of DTT modifies the BRET ratio of the positive and negative controls but does not modify the dynamic range (DR). However, DTT is not necessary for the BRET reaction to occur efficiently: as it acts as a boost for the Rluc long term emission.

## 3- The use of protease inhibitor.

In order to avoid cell damage during the assay procedure and timecourse experiments, an analysis was conducted to determine whether the use of aprotinin perturbs ratiometric measurements. Aprotinin is a protease inhibitor which is often used during cell culture to maintain cell integrity. Aprotinin at a concentration of 2  $\mu\text{g}/\text{ml}$ , did help maintaining cell viability over a time period of 2 hours. In the presence of aprotinin, viability decreased by less than 10% compared to more than 15% in absence of the protease inhibitor. During the same period of time, the BRET ratios were not affected significantly by the presence of aprotinin (data not shown).

#### 4- Effect of the pH.

The effect of cell media pH on BRET ratios was analyzed. For that purpose, we made stable transfections of Rluc alone and Rluc●EYFP cell lines. Stable Rluc and Rluc ●EYFP CHO cell lines were seeded using (PBS) media having different pH. Upon substrate addition, a BRET timecourse was started and the ratios were calculated. Figure 27 shows the effect of media pH on BRET ratios. The ratios tended to increase as pH decreased. This was true for the Rluc alone but not necessarily for the Rluc ●EYFP fusion. The best signal to noise was found at pH 7.1 which is close to the PBS pH (7.2). These results suggest that a pH stabilizer like HEPES could be used during drug screening and that the addition of certain compounds (eg. very acidic or basic peptides) could change the pH sufficiently to modify BRET detection.

#### 5- Effect of HEPES

Because pH stability is important in order to get meaningful ratio (see above), an analysis was conducted to determine whether Hepes could be used in a BRET assay without disrupting the energy transfer. Cells stably expressing Rluc or Rluc ●EYFP, were incubated during BRET detection in presence or absence of Hepes 10mM pH 7.2. The effect of Hepes in presence of DTT was also assayed. Figures 28 and 29 show the effect of Hepes on Rluc and Rluc ●EYFP fusion constructs, respectively. Hepes modified the ratio without disrupting it. The ratios were increased in presence of HEPES for both the Rluc and Rluc ●EYFP constructs, but DR did not change. Furthermore, there was an additional effect on the ratio when the assay was performed in the presence of DTT and HEPES, but again without changing the DR.

#### 6- Effect of DMSO

DMSO is often used as a solvent to dissolve the compounds and as a result could find its way into a given assay mixture. Various quantities of pure DMSO were added directly into wells of the microplate. Total volume was kept at 200 µl for each condition. Figure 30 shows the effect on the energy transfer

in presence of HEPES. It was observed that only a small effect resulted with Rluc emissions in the presence of DMSO (data not shown).

## **EXAMPLE XII: IN VIVO APOPTOSIS SENSOR**

Apoptosis or PCD (programmed cell death) is a normal cellular process found in most cell types. It is involved in many aspects of cell homeostasis and organism development (e.g. PCD is responsible of the modeling (shape) of an organ during morphogenesis) (Nicholson, D.W. and Thornberry, N.A., 1997, TIBS, 22: 299-306; Kinloch, R.A., et al., 1999, TIPS, 20: 35-42). Very often, inappropriate PCD is found in many diseases like cancer, neurodegenerative diseases, etc. PCD is a regulated way for cells to die at a specific time without stimulating the defense mechanism of the organism (e.g. inflammation) and hence distinct from the other type of cell death, necrosis, generally resulting from cellular accident.

Many proteins and lipids are involved in the PCD. Among them, the caspases which are part of a large family of cysteine proteases (Nicholson, D.W. and Thornberry, N.A., 1997, TIBS, 22: 299-306). Caspases recognize and cleave specific amino acid sequences (usually a sequence of 4-5 amino acids) and are part of a protein cascade that ends by activation of effectors involved in the degradation of protein (by activating general proteases) and DNA. Specific receptors at the plasma membrane e.g. fas-R, TNF-R) and their adaptor proteins trigger the apoptotic signal by the activation of a first line of caspases (caspase -2, 8, -10) depending of the cell type). These activated caspases then activate by cleaving a specific amino acid sequence, other caspases (-3, -7, -9, -4). These, in turn could activate other caspases and act also on cellular targets to generate the cell disruption. Apoptosis can be triggered also by chemical agents in some cell types (e.g. thapsigargin on HL-60 cell line or staurosporine on HeLa cell line).

A BRET apoptosis sensor was engineered by introducing a caspase-3 recognition site in the linker region of the Rluc-EYFP fusion construct. Upon induction of apoptosis, caspase-3 should recognize



and cleave the linker region which should separate Rluc of EYFP and thereby decrease the BRET ratio over time.

The caspase-3 site was introduced using annealed complementary oligonucleotides. The two complementary oligonucleotides encoding the caspase-3 site: Gly-Asp-Glu-Val-Asp-Gly. Only the Asp-Glu-Val-Asp used were: Oligonucleotide sense: 5' GATCCGGCCGACGAGGTGGACGGCGAATCCGCGGTAC and Oligonucleotide antisense: 5' CGCGGATTCGCCGTCCACCTCGTCCGGCCG

Once annealed, the oligonucleotides form a double strand DNA having two cohesive restriction sites at both ends: 5' BamHI and at 3' KpnI. pT7/Rluc-EYFP was digested using BamHI and KpnI and the annealed double oligonucleotide was subcloned into the pT7/Rluc-EYFP vector. The Rluc-EYFP and the Rluc-caspase-EYFP fusion genes from pT7 vector were then subcloned into the mammalian expression vector, pCEP4 (Invitrogen) using NheI and NotI restriction enzymes.

DNA sequences for the two constructs needed in this example are presented in Figures 57 and 59.

pCEP4/Rluc-EYFP and pCEP4/Rluc-caspase-EYFP DNAs were transfected into HeLa cell using LipofectAMINE (BRL/Gibco-Life technologies) in 100mm dishes as described in the manufacturer's protocol. 24 hours post-transfection, transfected cells were harvested and distributed into 96-well microplates (white Optiplate from Packard) at a density of 30,000 cells per well. The following day, cells are induced to undergo apoptosis using staurosporine 1mM dissolved in ISCOVE media (BRL/Gibco-Life technologies) without serum and phenol red at 37°C (total volume here is 100ml) for 5 hours. 50 ml of BRET buffer (PBS  $\text{Ca}^{2+}/\text{Mg}^{2+}$  + glucose without aprotinin) and 50ml of coelenterazine h (20mM) is added to start the bioluminescent reaction. Light emissions are detected using the BRETCount (read length sec for each filter, temperature 25°C). Data was collected 10 minutes after addition of coelenterazine h. The assay was done in quadruplicate.

Figure 31 gives representative BRET ratio changes in HeLa cells after 5 hours of apoptosis induction by staurosporine. A significant ratio change (0.1 unit) occurs when cells transfected with the apoptosis sensor were induced by staurosporine. Ratios decreased.

### EXAMPLE XIII: MINI-SCREEN ASSAY

A major application of BRET is drug screening in live cells. A target (in this example,  $\beta 2$  receptor) was stably transfected into mammalian cells (in this example, CHW) and co-expressed in combination with a transiently transfected sensor or a reporter gene (in this example, the cAMP sensor CBP:CREB). Cells were then seeded in 96-well microplate in a suitable buffer. Mini-screens were performed either with attached cells (on the bottom of the well) or using cell suspensions. If attached cells were used, a waiting period of at least several hours at 37°C was allowed for so that cells could adhere to the bottom of the wells. Once adhered, test compounds were added to the cells and incubated at a suitable temperature for the assay (generally between 15-42°C). Generally but not necessarily, compounds were dissolved in an organic solvent (e.g. DMSO). Compounds can be pooled before addition to the wells. Coelenterazine was then added to start the bioluminescent reaction, followed by emissions detection.

While in this example, the cells were transiently transfected with the sensor in a stable cell line expressing the receptor, alternative embodiments may use cells transiently transfected with target in a stable cell line expressing sensor, or a cell line stably expressing both the receptor (target) and the sensor.

48 hours post-transfection, transfected CHW-b2 were seeded at a density of 50 000 cells per well in a white Optiplate (Packard). 10 ml of different compounds dissolved in DMSO at various concentrations, were added in each well to a total volume of 150ml (cell+buffer+compound). The first row on the microplate was dedicated for the positive control (isoproterenol) and the negative control (DMSO). The assay can be conducted as a "blind test", such that the assayer does not know the identity of the compounds being tested and a second person prepares a "mother plate" containing each of the compounds at two different concentrations. The "blind" assayer takes 10ml from each well from the mother plate and transfers the samples to the assay plate containing the cells. 50ml of coelenterazine

h (20mM) was then added to each well to start the bioluminescent reaction. Microplate was then loaded in the BRETCount for light measurement, as described above, at 25°C. Data was collected 68 minutes after addition of coelenterazine. False positives may be identified by repeating the assay with the mother cell line CHW, transfected with the sensor but which do not express b2.

It should be apparent to one skilled in the art, that alternative embodiments falling within the scope of the invention and employing other drug screening protocols may be adapted to employ the BRET assay.

Figure 32 represents the results from a mini-screen assay ratio 550/470 nm vs compound position in the well. The first four wells of row 1 are the negative control (DMSO alone) (N). The four following wells are the positive control (P). Median line was calculated by the following formula: Ratio positive (obtained with control positive) minus ratio negative (obtained with the negative control) divided by 60 multiplied by 100. For this particular assay the median line was settled at 2.029. All ratio values from the other wells, higher than the median line, are considered as hits (1-5). The identify of all the reactive compounds that interacted with b2 receptor are: 1: forskolin 2 mM; 2: epinephrine 20nM; 4 forskolin 2mM).

#### EXAMPLE XIV: AN ALGORITHM FOR THE BRET ASSAY

An algorithm was developed to decrease mathematically basal level during BRET assay. Although there other algorithms that could also be used, one simple approach, is to remove the portion of Rluc emission in the EYFP peak. For example, this could be calculated using the simple formula:

$$\frac{(\text{Em550} - \text{Em470} \times \text{correcting factor})}{\text{Em 470}} = \text{corrected ratio}$$

where Em550 is the emission value (CPS) obtained with the filter 550nm (filter for EYFP emission)  
Ex470 is the emission value (CPS) obtained with the filter 470nm (filter for Rluc emission)

The correcting factor was experimentally calculated using Rluc construct transfected alone (without the EYFP construct). The correcting factor is the ratio (550/470nm) of the Rluc construct at a given time after addition of coelenterazine. In the following example, Figures 33 and 34 represent the non corrected and corrected data, respectively. The same raw data (light emissions using both filters)) were used in both figures. The only difference is the that in Figure 34 the ratio have been corrected using the above formula. In this example, the above algorithm do not change the range dynamic of the assay which still approximately 0.1 (the difference between maximal and minimal ratio) in both figures. But the SNR (signal to noise ratio) does change. SNR is the ratio between maximal and minimal BRET ratio values. In Figure 33, the SNR is 1.07 compared to 4.77 for Figure 34. Introduction of algorithm in calculating BRET ratios, could be very useful during HTS process since it decreases background and increase SNR therefore ease result interpretation.

#### **EXAMPLE XV: VARIOUS PARAMETERS THAT AFFECT THE ASSAY (LUMICOUNT)**

This example summarizes various experiment performed to analyze BRET in mammalian cells. The experiments were divided in two parts: 1) determination coelenterazine permeability; 2) BRET feasibility using the LumiCount.

##### **Determination of Coelenterazine permeability**

- i) **Coelenterazine derivatives.** Using the plasmid pRluc-CMV (Promega) and the LumiCount, it was determined which of the coelenterazine derivatives gives the highest signal. Cells (HeLa) were transiently transfected in T-75 flask with pRluc-CMV or control vector using LipofectAMINE. At 48 hours post-transfection, cells were harvested and seeded in 96-well white ViewPlate (in duplicate). Coelenterazine derivatives (1 mM) were then added in each well. Luminescence from transfected cells was measured using the LumiCount 4 minutes after addition of coelenterazine derivatives. LumiCount was set at 1100V PMT and 1 sec read length.

**Table IV: Assay of coelenterazine derivatives**

Coelenterazine derivatives	Normalized RLU
native	2030
n	35936
f	180126
h	173547
hcp	48144
control vector (without Rluc gene)	855*

\*This value is elevated because of cross-talk between wells.

The electronic background is about 20-40 RLU. An evaluation of the cross-talk was determined in the LumiCount at 1.3% in the well next to the emitter and at 0.2% the second well next to the emitter. The cross-talk increased to 3% when filters (see below) were used.

- ii **Cell linearity experiment.** Cells (HeLa and CHO) were transiently transfected in T-75 flasks with pRluc-CMV using LipofectAMINE. At 48 hours post-transfection, cells were harvested and seeded (in quadruplicate) in 96-well white ViewPlate at different cell densities (ranging from 0 to 150,000 cells per well). Coelenterazine h in PBS (1 mM) was then added in each well. Luminescence from transfected cells was measured using the LumiCount, 10 minutes after addition of coelenterazine. LumiCount was set at 1100V PMT and 0.1 sec read length.

Figure 35 presents the results observed. As is evidenced, Rluc luminescence is linear over the range of cell number analyzed. Very high signal to noise (SNR) ratios (over 200) could be obtained using moderate amount of cells (50,000 cells per well). Even higher RLU and SNR could be achieved if the analysis was performed during the Rluc flash of luminescence. The slopes for HeLa and CHO cells are different which presumably reflects the differences in transfection efficiencies between the two cell lines and/or differences in CMV promoter activity between the two cell lines.

- iii) **Time-course experiment.** Cells (HeLa and CHO) were transiently transfected in T-75 flasks with pRluc-CMV using LipofectAMINE. At 48 hours post-transfection, cells were harvested and seeded in 96-well white ViewPlate at a density of 50 000 cells per wells (in quadruplicate).

Coelenterazine f (1 mM) was then added in each well. Luminescence from transfected cells was measured using the LumiCount at different times after addition of coelenterazine. LumiCount was set at 1100V PMT and 0.1 sec read length.

Figure 36 shows the results of these Time-course studies of Rluc luminescence. In both cell lines, decay of Rluc luminescence is rapid after addition of coelenterazine f. This experiment was repeated with native coelenterazine and similar results were obtained. The half-life of the reaction is 11 minutes for CHO cells and 13 minutes for HeLa cells. These values are similar to what was found in literature for *in vitro* (cell extracts) experiments.

- iv) **Dose-response of coelenterazine derivatives.** HeLa cells were transiently transfected in T-75 flasks with pRluc-CMV using LipofectAMINE. At 48 hours post-transfection, cells were harvested and seeded in 96-well white ViewPlate at a density of 25 000 cells per well (in quadruplicate). Coelenterazine f or h was then added in each well at different concentrations. Luminescence from transfected cells was measured using the LumiCount, 3 minutes after addition of coelenterazine. LumiCount was set at 1100V PMT and 0.1 sec read length.

Figure 37 shows the results of the experiments for a dose response of coelenterazine derivatives. Saturations were obtained for both coelenterazine derivatives. From these curves, the  $K_m$  of Rluc for both coelenterazines were calculated. These are 1.2 and 0.8 mM for coelenterazine f and h respectively. Again, these values are similar to what was found in literature for *in vitro* (cell extracts) experiments.

- v) **Dose response using lysed and whole cells.** HeLa cells were transiently transfected in T-75 flasks with pRluc-CMV using LipofectAMINE. At 48 hours post-transfection, cells were harvested, washed and divided in two. One fraction of the cells were directly seeded in 96-well white ViewPlate at a density of 25 000 cells per wells (in quadruplicate). Cells in the other fraction were lysed using a Dounce potter and lysing buffer (hypotonic solution). After potting, unlysed cells and nuclei were removed by centrifugation. The lysed extract was distributed in the 96 well white plate. Coelenterazine h was then added in each well at different concentrations. Luminescence

from transfected cells was measured using the LumiCount, 2-3 minutes after addition of coelenterazine. LumiCount was set at 900V PMT and 1 sec read length.

Figure 38 shows the results of a comparison of lysed and non-lysed cells. Both assays gave approximately the same  $K_m$  (around 1mM) and similar absolute RLU meaning that coelenterazine cross rapidly and massively the cell membrane.

The following studies pertain to the BRET assay.

#### **i) Fusion type assay**

Using the above parameters for Rluc luminescence and knowing that cell membrane is permeable to coelenterazine, it was determined that one should start to assay BRET in mammalian cells. The Vanderbilt University constructs were subcloned into mammalian expressing vectors (pCDNA3.1). BRET was assayed using initially the fusion-type vector where the Rluc protein was fused to EYFP (third construct in annex 1). For that purpose, the LumiCount was modified by increasing the length between the PMT and the microplate. This was done in order to put a filter on the microplate. To measure BRET, the luminescence is detected at two wavelengths: 480nm and 530nm (using two 50 X 50mm filters from Ealing). Hence, after addition of coelenterazine on cells, the 530nm filter was fixed on the microplate using Fun-Tak (from Lepage) and the luminescence from the microplate was read. Then the 530nm filter was replaced with the 480 filters and luminescence was again read. Ratios of emission at 530nm and 480nm were calculated in order to quantify BRET.

The cells (CHO) were transiently transfected in T-75 flask with pcDNA3.1-Rluc + pcDNA3.1-EYSP or pcDNA3.1-Rluc + pcDNA3.1-Rluc • EYSP (fusion) using LipofectAMINE (fig. 41). At 48 hours post-transfection, cells were harvested and seeded in 96-well white ViewPlate at a density of 50,000 cells per well (in quadruplicate) in 50 ml of PBS. The modified LucLite buffer (100ml) was then added before adding 50 ml of coelenterazine f (final concentration: 1 or 5 mM). Luminescence from transfected cells was measured using the LumiCount at different times after addition of coelenterazine.

LumiCount was set at 1100V PMT and 1 sec read length. Table V shows a summary of the results from different transfections.

Table V: BRET assay using the LumiCount (\* N>10 different transfections)

Constructs	Ratio 530/480
Control Rluc*	0.6-07
Control Rluc+EYFP (cotransfected)*	0.7-1.0
Fusion Rluc:EYFP*	1.2-1.6
Rluc-kaiB:EYFP-kaiB (positive)	1.2-1.45
Rluc-kaiB (negative control)	0.8
Rluc-kaiB+EYFP-kaiA (negative control)	0.86-0.91

BRET can be detected using the LumiCount. The ratios from the fusion Rluc::EYFP are always higher compare to both negative controls. Note that the ratios do not seem to be stable over time (see Figure 39 and 40) and the ratios from negative controls were higher than expected especially when using higher coelenterazine concentrations.

- 1) Ratio stability. BRET or ratios were stable if Rluc emission was stable. But results were variable if Rluc emission was not stable. When using coelenterazine h, ratios from negative control increase with time and ratios from the fusion decreased to a point where both curves crossed each other (Figure 39). This point occurred latter in time when lower concentration of coelenterazine was used. When native coelenterazine was used, ratios were more stable (see figure 40).
- 2) High ratios for negative control. According to fluorescence scan in bacteria (Vanderbilt's results) the negative control should have a ratio of 0.5-0.6. This is less than obtained in mammalian cells.

Figure 39 shows the results of studies of ratio stability when using coelenterazine h in LucLite. Both problems are related. They are dependent of coelenterazine (type and concentration) and probably of number of sensor molecules per cells. To verify new assays were performed using less DNA during the transfections (Figure 41 and 42) or by lysing the cells to decrease the entropy of the reaction: sensor molecules dilution in higher volume (Figure 43). The volume of the cells is very small (high entropy). When too much of Rluc is emitting (because of its high expression or the use of the strong coelenterazine



derivatives), nearby EYFP in the negative control is activated resulting in a increase of the ratio. Hence, light output at 530nm increase so the ratio. If there is high levels of Rluc or EYFP molecules and/or coelenterazine, activation of EYFP (nearby) from Rluc, emission is stronger. In fact, by comparing the negative control Rluc and the negative control Rluc+EYFP (see table 2), ratios from the latter were usually higher indicating that some non-specific BRET (radiative energy transferred) occurred. Figure 40 shows results of ratio stability when using native coelenterazine in LucLite.

Figure 41 shows BRET detection in cells transfected with 10 times less DNA. Figure 42 shows BRET detection in cells transfected with 20 times less DNA. By using less DNA during the transfections, the Rluc+EYFP negative control ratio was diminished to the level of Rluc alone, indicating that there is less non-specific BRET occurring in the Rluc+EYFP negative control. Furthermore the ratios were more stable over time when expressing lower amount of molecules. The difference between fusion and negative control were higher when using less DNA hence increasing the dynamic range of the assay. Finally from a biological point of view, expressing lower amount of sensor molecules within the cells is better: it will cause less disturbance to the "normal" metabolism of the cell.

The best conditions in terms of the stability of ratio and non-specific BRET produced, were found when the cells are lysed (Figure 43). In these conditions, all the ratios are stable and all the negative controls were equivalent. Figure 43 shows the results of BRET detection when cells are lysed. Transfections were done using the "normal" amount of DNA.

## **ii) Protein-protein type assay.**

Protein-protein assay using kai domains was also performed (see table 2). Only Rluc-kai B/EYFP-kaiB interaction resulted in higher ratio than Rluc-kai B/EYFP-kai A control.

## **iii) Formatting of the assay.**

**Assay media.** It was determined whether the assay media had a effect on Rluc emission (Figure 44). The above experiments were repeated using PBS or DMEM/F12 (without phenol red) media and

LucLite. For both the fusion and the negative control, PBS gave the highest Rluc emission. Figure 44 shows the results of the comparison between PBS and DMEM/F12 (without phenol red) for assaying BRET.

**Other conditions tested.** Different types of microplates and assay volumes were tested as to whether they had an effect on BRET. The volume used for the assay did not influence the ratio. The type of microplate (black or white) did influence the ratio: black microplate decreased the ratios compared to white plate. But this happened for both the negative control and the fusion.

Table VI: Microplate type and volume effect on BRET

Construct/Conditions	Ratio 530/480
Rluc+EYFP White microplate vol. 200ul	0.87
Rluc●EYFP	1.31
Rluc+EYFP Black microplate vol.200ul	0.76
Rluc●EYFP	1.04
Rluc+EYFP White microplate vol. 100ul	0.90
Rluc●EYFP	1.32
5 uM coelenterazine 50,000 cell per well.	

The dynamic range (number of folds over negative control) is somewhat small being about two, which was not that different from literature. Usually for FRET assay, the dynamic range was between 2 and 3 times.

#### EXAMPLE XVI: VARIOUS PARAMETERS THAT AFFECT THE ASSAY (BRET COUNT)

The following experiments pertain to factors affecting the stability of the assay. During the course of the BRET study, it was determined that Rluc emission in live cells was not stable over time having a half-life of about 5-8 min. Rluc emission stability important for BRET assay in order to achieve higher reproducibility (tighter CV, SD) and easier adaptation to HTS. Initially, the modified LucLite buffer was used to stabilize the Rluc emission with little success. This buffer which does not contain the Triton N-101 detergent, but still contains DTT, had some stabilization effect by doubling the Rluc emission half-life (12-16 min). But the use of this modified LucLite, is not recommended in live cell assay because long term exposure damaged the cells: solutions are not isotonic. Beside or in combination with the modified LucLite buffer, different assay conditions were tried in order to obtain the better stability. Among them,  $\text{Ca}^{2+}/\text{Mg}^{2+}$  dependency, amount of DNA transfected, cell concentration, coelenterazine concentration, type of media (PBS vs DMEM), time of incubation of the modified LucLite with the cells. Beside DTT, two observations came out from this previous study for the stabilization of Rluc: 1- coelenterazine concentration was the only conditions that had a affect (small though) on the half-life on the reaction. 2- true glow (half-life over 1 hour) was achieved only if the cells were lysed. The stability (half-life) of the Rluc even in lysed cells, is different compared to FFluc. FFluc half-life is more than 3 hours compared to 1 hour for the Rluc when they were assayed using the LumiCount and the DuoLite (DuoLite was later renamed FireLite). The main objective here is to find assay conditions that will allow stable Rluc emission and that those conditions will not interfere with the energy transfer of BRET. The followings are the conditions that were tested:

- 3- **Time-resolved emission analysis.** Rluc emission is divided into two phases. Within the first 20-30 minutes the emission decays rapidly without hitting the zero. After 30 minutes Rluc emission is more stable. It needed to be determined whether this "stable" state could be use for HTS: it is caused by the autoluminescence of the coelenterazine and if not, is the height of the curves are linear with the Rluc content of the cell.
- 4- **Temperature effects.** The effect of temperature on Rluc emission and on BRET ratio is to be assayed.

- 5- **Analogu effect.** If available, the use of inactive coelenterazine analogues and/or coelenteramide (coelenteramide is a by-product of the reaction) would be tried for the stabilization of Rluc.
- 6- **Radical scavenger effect.** Finally, the effect of the cell permeable radical scavenger: DTT.

Two compounds found in the LucLite solutions are important for the stability of FFluc emission: DTT and AMP. DTT at high concentration seems to act as a radical scavenger and functions by limiting the availability of  $O_2$ . At very high concentration, DTT even increases emission of photons through an unknown mechanism.  $O_2$  is essential for the FFluc reaction as are ATP,  $Mg^{2+}$  and the FFluc substrate, luciferin. Figure 45 shows DTT effects on the Rluc emission stability.

FFluc oxidation reaction is divided in two steps. First, there is the formation of a "activated intermediate" when luciferin and ATP bind the enzyme and when the ATP is degraded into AMP which in turn forms a reactive anhydride. Adding AMP to the reaction slows the formation of the activated enzyme and so the turnover of the reaction making it more slower. In the second step, the activated intermediate reacts with  $O_2$  and breaks down into  $CO_2$ , oxyluciferin and green-yellow light at 560nm. Others components of the LucLite kit are also important (to a lesser degree though) for the emission stability of FFluc: oxalic acid and phenylacetic acid as protease inhibitors, EDTA for the control of  $Mg^{2+}$  concentration and Hepes buffer for a tight control of the pH (independent of the  $CO_2$  content of the media). Promega (Madison, WI) also adds in the FFluc kit D-analogue substrate and CoA (see below).

The enzymatic mechanism of Rluc is different compared to FFluc. First, Rluc uses the coelenterazine as substrate and produces blue light at 470-480nm. It is not a two step process: there is no requirement for ATP and  $Mg^{2+}$ . The turnover of the reaction is faster than for FFluc. Because it is not a two step process Rluc reaction suffers from autoluminescence of its substrate. Therefore, Rluc reaction is less sensitive than FFluc reaction. The levels of the autoluminescence is greatly influenced by the availability of hydrophobic microenvironments to protect the luminescent excited state from the water. Hence, the presence of hydrophobic proteins, cellular debris (membrane), detergent micelles

can diminish sensitivity. On the other hand, the coelenterazine is membrane permeable making Rluc attractive for cell based assays. Finally, it seems that DTT is also effective to stabilize Rluc acting probably as radical scavenger. Note that DTT is also membrane permeable at concentration higher than 1 mM.

From a structural point of view, both enzymes are very different. Rluc comes from a coelenterate and has a MW of 36 kDa. FFluc comes from the insect and has a MW of 61 kDa. There is no obvious amino acid sequence identity between them. The amino acid sequence of the FFluc is more related to a diverse family of acyl-CoA synthetase. That emphasized the importance of CoA presents in Promega's kit, to stabilize the FFluc. Hence, both enzymes are totally different both structurally and enzymatically (substrate, requirements for the reaction, etc.)

#### **Methods:**

Transfections: CHO-K1 cells were transiently transfected using LipofectAMINE (BRL/Gibco) in 100mm dishes according to manufacturer's protocol (0.2ug for each construct + X carrier DNA for a total of 8ug). Transfection efficiencies between 40-60% were obtained using this technique (when analyzed using a GFP reporter vector and fluorescence microscope). 48 hours post transfection, cells were harvested using low concentrated trypsin, counts and diluted in D-PBS (containing Ca/Mg). For most experiment, 50,000 cells per well were used in white Optiplate. Assay were performed in quadruplicate. The modified LucLite buffer was used initially to stabilize Rluc emission. Light emission was triggered by the addition of coelenterazine h 5 uM (50ul of a 20 uM stock).

The experiments were conducted using the BRETCount at various time after addition of the coelenterazine. Parameters used for the BRETCount were: luminescence SPC mode, read length 1 sec for each wavelengths at 25°C, filters: 470DF60 550DF80. In some experiments, results were obtained with the LumiCount. Parameters for the LumiCount were: read length of 0.5sec PMT 1100V.

#### **1- Time resolved Rluc emission.**

Cells were transfected with the Rluc construct only. Two days after transfection, cells were harvested and seeded at different concentration in the modified LucLite buffer. Coelenterazine h was added before the detection in the BRETCount. Figure 46 shows Rluc emission stability over time.

Figure 46 shows that starting at 10 minutes after addition of coelenterazine, Rluc emission tend to stabilize over time. Furthermore, even after 10 minutes, it was noted that RFU levels still dependent of cell number. In order to be sure, was repotted the graph by using RFU vs cell number at different fixed times (see Figure 47).

Figure 47 shows Rluc emission levels versus cell number at fixed time. Figure 47 shows that Rluc emissions were linear with cell number even at 58 minutes post induction ( $t=58$ ). This is a surprise since at this time, the enzyme is not anymore in the optimal conditions to work properly (i.e. substrate concentration is limited, high amount of by-product present in the reaction). Furthermore, the slopes at  $t=30$  and  $t=58$  were similar meaning that Rluc emission half-life does not change substantially during this time period. The experiment was repeated using different relative amount of transfected cells but with the same total amount of cells. The transfected cells were mixed with untransfected cells to achieve a total cell number of 100,000 cells per well. The rational is that coelenterazine autoluminescence is cell dependent (in fact lipid dependent). Hence increased cell numbers per well will contribute to the linearity seen in Figure 47. To verify this assumption, the experiment was repeated using the same total number of cells per well. Figure 48 shows the Rluc light emissions using different amount of transfected cells mixed with untransfected cells to make a total cell number of 100,000 cells. These mixed populations were then seeded into the microplate just before starting the reaction with the coelenterazine.

Figure 48 shows Rluc emission stability in presence of a fixed number of cells. Figure 49 shows Rluc emission levels vs cell number at fixed time.

Figures 47 and 49 show that Rluc emission levels are linear with cell number even at 40 minutes post-coelenterazine addition. Therefore, the Rluc light outputs observed at 30 minutes and beyond, do not

result from autoluminescence of the coelenterazine but from the true Rluc enzymatic activities and these latter are linearly dependent of Rluc content of the cells.

- 2- **Temperature effects.** Using the LumiCount, it was determined that at lower temperature, Rluc emission was reduced and stabilized over time. These results are summarized in Figure 50 and 51. Figure 50 shows that at 15°C the Rluc emission is stabilized over the period of time analyzed. The BRET ratio (Figure 51) appears to change with assay temperature: it decreases when temperature is decreased. At 10°C, the Rluc emission is too much reduced to be workable using the LumiCount (data not shown).

Figure 50 shows the effect of the temperature on the Rluc emission. The LumiCount and the reagents needed for the assay were stored in the walk-in refrigerator 2-3 hours before starting the experiment and the internal LumiCount temperature was settled either at 15 or 10°C. Figure 51 shows the effect of the temperature on the BRET ratio (LumiCount).

Further characterization of the effect of temperature using the BRETCount in which the internal temperature is more stable and controllable compared to the LumiCount (Figure 52). The experiments were repeated using the BRETCount at different temperature. The highest SNRs were obtained at the assay temperature of 19°C. The ratio tends to increase when temperature increases which confirms the results obtained with the LumiCount. Above 19°C, even the ratios of the negative controls (Rluc+EYFP) increase with temperature. Figure 52 shows the Effect of the temperature on the BRET ratio (BRETCount).

- 3- **Radical scavenger effect.** The BRETCount was used (which has a higher through-put compared to the LumiCount) to analyze the effect of radical scavenger, more particularly the DTT, on both the Rluc emission and the BRET ratio. It has been previously shown that the DTT helps to stabilize the Rluc emission (see Figure 53).

Figure 53 shows the effect of the DTT on the Rluc emission. Harvested cells were diluted into PBS (+Ca<sup>2+</sup>/Mg<sup>2+</sup>) containing different concentration of DTT. Figure 53 shows the effect of the DTT (in

PBS  $\text{Ca}^{2+}/\text{Mg}^{2+}$ ) on the Rluc emission in live cell. Increased concentration of DTT stabilizes the Rluc emission by lowering the initial emission flash of the enzyme. The highest stability was obtained at 100mM of DTT. At this concentration, the DTT decreases the viability of the cells. Viability analysis was performed using the trypan blue dye at different time after the cells were diluted in the buffer containing the DTT. 10mM DTT was chosen which gives some (not that high though) Rluc stability and good long term viability (below 15% loss over 2 hours).

Figure 54 shows the effect of the DTT on the Rluc emission and the BRET ratio (BRETCount). These results were obtained with the stable cell lines expressing Rluc only (negative control). PBS=PBS+ $\text{Ca}^{2+}/\text{Mg}^{2+}$ +glucose. DTT 10mM. Figure 55 shows the effect of the DTT on the Rluc emission and the BRET ratio (BRETCount). These results were obtained with the stable cell lines expressing the fusion Rluc::EYFP (positive control). PBS=PBS+ $\text{Ca}^{2+}/\text{Mg}^{2+}$ +glucose DTT 10mM.

Figures 54 and 55 show that DTT effect the BRET ratio. In PBS ( $\text{Ca}^{2+}/\text{Mg}^{2+}$ +glucose) alone, BRET ratios are lower than in presence of DTT. When DTT is added, ratios increase by almost 0.1 BRET unit. The ratio of the negative control also increased. Hence, the DTT do not affect (or has a small effect) the energy transfer.

It was determined that the assay temperature has a effect on the BRET ratio. This observation is important in the context that most biological processes which will be assayed using BRET, are performed at 37°C.

To a certain point, DTT helped to stabilize the Rluc emission. Good stabilization occurs at the emission at 100mM DTT which at this concentration cells were damaged making this concentration are not suitable for cell-based assay. At 10mM, it is noted that DTT has also a small effect on the BRET ratio and not only on the yield and duration of Rluc emission. In fact it appears that DTT changes the spectral characteristic of the Rluc emission to a small degree. The emission would be right shifted on the emission scan.



Time resolved emission analysis revealed that 30 minutes after addition of coelenterazine, the Rluc emission entered a relatively stable phase. This phase lasted for more than 60 min (data not shown).

This invention provides The components of this system interact to influence the spatial relationship between the bioluminescent protein and the fluorophore, that is demonstrated by the light emission from the system. The modulator can be a single entity, covalently attached to both the bioluminescent protein and the fluorophore as demonstrated in Figure 1, it can be two separate entities, each linked covalently to either the bioluminescent protein or the fluorophore as demonstrated in Figure 2, or an alternative configuration that falls within the scope of the invention. This system can be used in both *in vivo* or *in vitro* assays to detect molecular changes in a wide variety of applications, and is amenable to automation. In particular, it is useful for assaying protein interactions, enzyme activities and the concentration of analytes or signaling molecules in cells or in solution.

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## WE CLAIM

1. A bioluminescence resonance energy transfer (BRET) system comprising: 1) a bioluminescent protein that has luciferase activity; 2) an acceptor fluorophore that can accept the energy from the bioluminescent protein when they are associated, in the presence of the appropriate substrate; 3) a modulator that influences the proximity or the orientation of the bioluminescent protein and the fluorophore, and 4) an appropriate substrate to activate the luciferase activity of the bioluminescent protein.
2. A system as in claim 1, wherein the luciferase and the fluorophore are attached to the same modulator.
3. A system as in claim 1, wherein the luciferase and the fluorophore are attached to separate modulator entities.
4. The system as in claim 1, wherein conformational change in the modulator causes a decrease in the proximity of a luciferase and a fluorophore.
5. The system as in claim 1, wherein conformational change in the modulator causes an increase in the proximity of a luciferase and a fluorophore.
6. The system as in claim 1, wherein conformational change in the modulator causes a change in the orientation of a luciferase and a fluorophore.
7. A system as in claim 2, which is a fusion protein construct comprising a luciferase attached to modulator which is attached to a fluorophore.
8. A fusion protein construct as in claim 7, wherein the fluorophore is a green fluorescent protein that is red-shifted in its excitation and/or emission spectra compared to wild type green fluorescent protein.

9. A fusion protein construct as in claim 8, wherein the fluorophore is a green fluorescent protein selected from the list comprising EYFP variants, EGFP, GFP wild type, GFPS65T, YFP, Topaz.
10. A fusion protein construct as in claim 7, wherein a target sequence is inserted into the amino acid linker sequence.
11. A fusion protein construct as in claim 10, wherein the target sequence is selected from the list comprising: a protease site, a phosphorylation site, a  $\text{Ca}^{+2}$  binding site, a cAMP binding site, an IP3 binding site, and a cGMP binding site.
12. A fusion protein construct as in claim 11, wherein the fluorophore is a green fluorescent protein that is red-shifted in its excitation and/or emission spectra compared to wild type green fluorescent protein.
13. A fusion protein construct as in claim 12, wherein the fluorophore is a green fluorescent protein selected from the list comprising EYFP variants, EGFP, GFP wild type, GFPS65T, YFP, Topaz.
14. A recombinant DNA encoding the fusion protein construct of claim 7.
15. A fusion gene that comprises the recombinant DNA of claim 14.
16. A recombinant DNA encoding the fusion protein construct of claim 10.
17. A fusion gene that comprises the recombinant DNA of claim 16.
18. A DNA cassette comprising a promoter operably linked to one or more fusion protein genes of claims 15 or 17.

19. A vector comprising a fusion gene as in claim 18.
20. A host cell transformed or transfected by the vector of claim 19.
21. A host cell as in claim 20, wherein the cell is human, mammalian, bacterial, or yeast.
22. A vector as in claim 19, wherein the gene construct is under the control of a constitutive promoter.
23. A vector as in claim 22, wherein the constitutive promoter is selected from the list comprising the following promoters and their variants and derivatives: CMV, SV40, RSV, EF-1 $\alpha$ , Tk, AdML, when the cell to be transformed or transfected is mammalian.
24. A vector as in claim 22, wherein the constitutive promoter is selected from the list comprising the following promoters and their variants and derivatives: T7, AraBAD, trc, pL, tac, and lac, when the cell to be transformed or transfected is a bacterial cell.
25. A vector as in claim 22, wherein the constitutive promoter is selected from the list comprising the following promoters and their variants and derivatives: nmt1, gal1, gal10, TEF1, AOX1, GAP, and ADH1, when the cell to be transformed or transfected is a yeast cell.
26. A virus comprising a fusion gene as in claim 18.
27. A host cell infected by the viral vector as in claim 26.
28. A virus as in claim 26 selected from the list comprising: retrovirus, adenovirus, Semliki and Sinbis and their variants and derivatives.
29. A virus as in claim 26, when the cell to be infected is an insect cell.

30. A vector as in claim 22, wherein the constitutive promoter is baculovirus, when the cell to be transformed or transfected is an insect cell.
31. A vector as in claim 19, wherein the vector is under the control of an inducible promoter.
32. A vector as in claim 22, wherein the inducible promoter is selected from the list comprising GRE, Tet-off, Tet-on, HSP, MMTV based promoter, and metallothioneine.
33. A system as in claim 3, which comprises:
  - (a) a fusion protein construct comprising a luciferase attached to modulator entity that is a protein or protein domain; and
  - (b) a fusion protein construct comprising a fluorophore attached to a modulator entity that is a protein or protein domain; and wherein
  - (c) the protein or protein domain in (a) is capable of interacting with the protein or protein domain in (b).
34. A fusion protein construct as in claim 24, wherein the fluorophore is a green fluorescent protein that is red-shifted in its excitation and/or emission spectra compared to wild type green fluorescent protein.
35. A fusion protein construct as in claim 25, wherein the fluorophore is a green fluorescent protein selected from the list comprising EYFP variants, EGFP, GFP wild type, GFPS65T, YFP, Topaz.
36. A recombinant DNA encoding one of the fusion protein constructs of claim 34.
37. A fusion gene encoding the recombinant DNA of claim 36.
38. A vector comprising a fusion gene as in claim 37.

39. A host cell transformed or transfected by a vector of claim 38.
40. A host cell as in claim 39, wherein the cell is human, mammalian, bacterial, or yeast.
41. The use of the system of claim 1 for second messenger screening.
42. The use of the system as in claim 41, wherein the second messenger is selected from the list comprising: cAMP, cGMP, Ca<sup>2+</sup>, IP<sub>3</sub>, NO, DAG, ceramide and derivatives thereof, and arachidonic acid and derivatives thereof.
43. The use of the system as in claim 1 for analyte screening.
44. The use of the system as in claim 43, wherein the analyte is selected from the list comprising: K<sup>+</sup>, H<sup>+</sup>, Ca<sup>2+</sup>, CO<sub>2</sub>, Na<sup>+</sup>, Cl<sup>-</sup>, Mg<sup>2+</sup>, O<sub>2</sub>, HCO<sub>3</sub>, NO, and ATP.
45. The use of the system of claim 1 for drug discovery.
46. The use of the system of claim 1 for drug screening.
47. The use of the system as in claim 1 to detect changes in protein-protein interaction.
48. The use of the system as in claim 1 in functional genomics to determine the cellular function of a gene by determining its binding partner.
49. The use of the system as in claim 1 in toxicology to measure the presence and concentration of toxic compounds.
50. The use of the system as in claim 1 in diagnosis.

51. The use of the system as in claim 1 in genotoxicity to measure the effect of a toxic compound on genome stability.
52. A system as in claim 3, wherein a luciferase is attached to a known protein, a fluorophore is attached to the products of a gene library, and an association between a known protein and a product of the gene library is detected by measuring resonance energy transfer between the luciferase and the fluorophore.
53. A kit comprising cells and reagents and instructions for performing BRET assays *in vivo*.
54. A kit comprising purified luciferase and a fluorophore, or cell extracts from transfected or transformed cells to perform an *in vitro* assay.
55. The use of the system in claim 1 for *in vitro* assays.
56. The use of the system in claim 1 for *in vivo* assays.

FIGURE 1

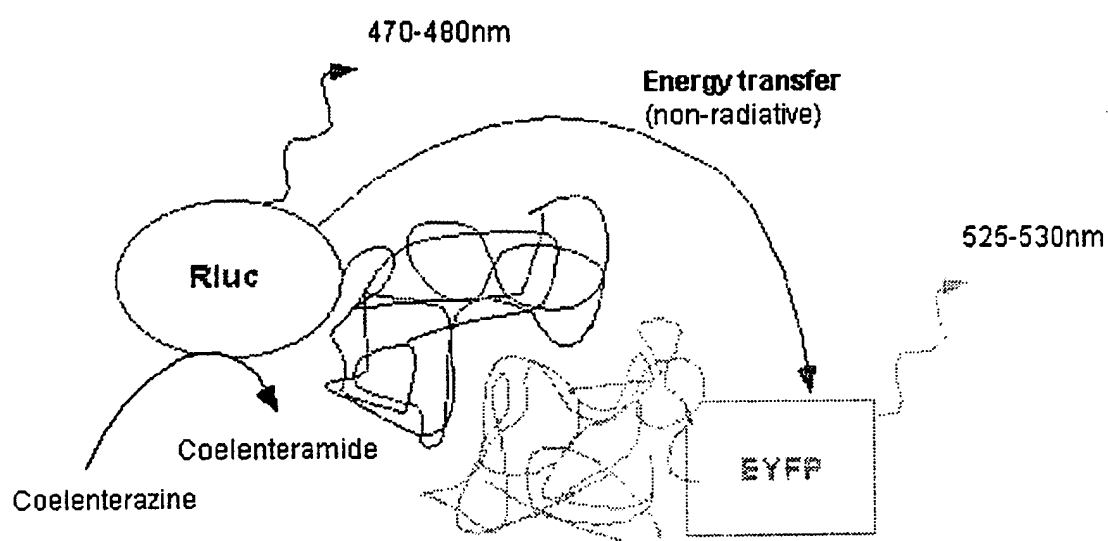




FIGURE 2

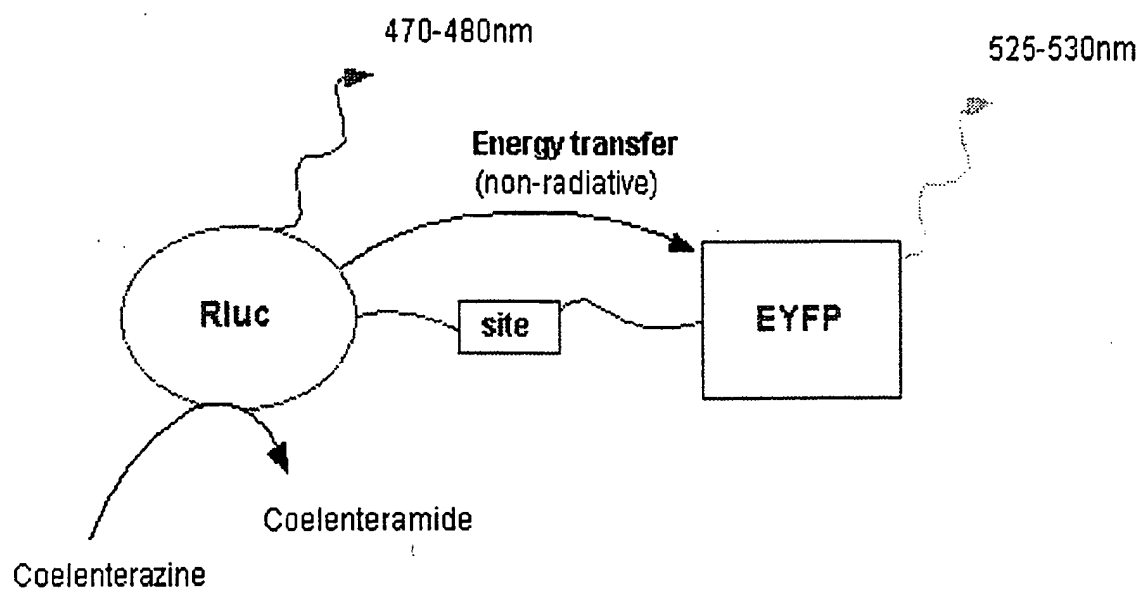


FIGURE 3

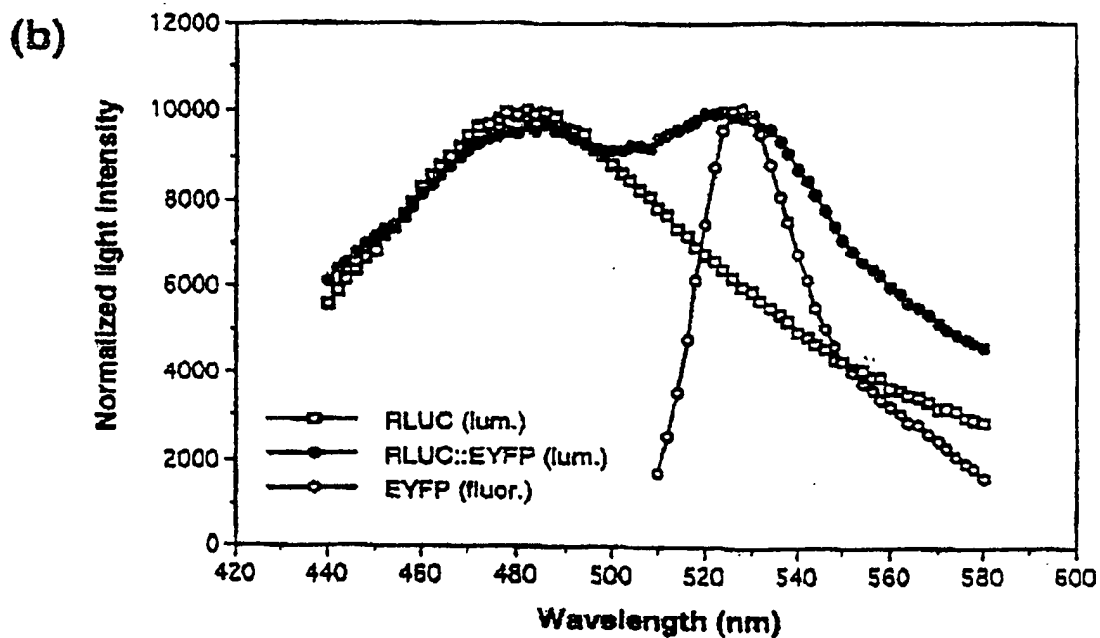
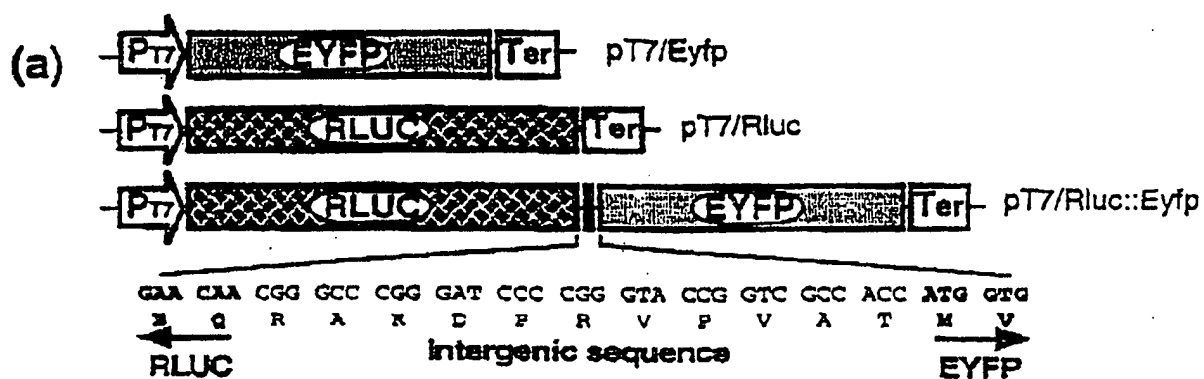


FIGURE 4

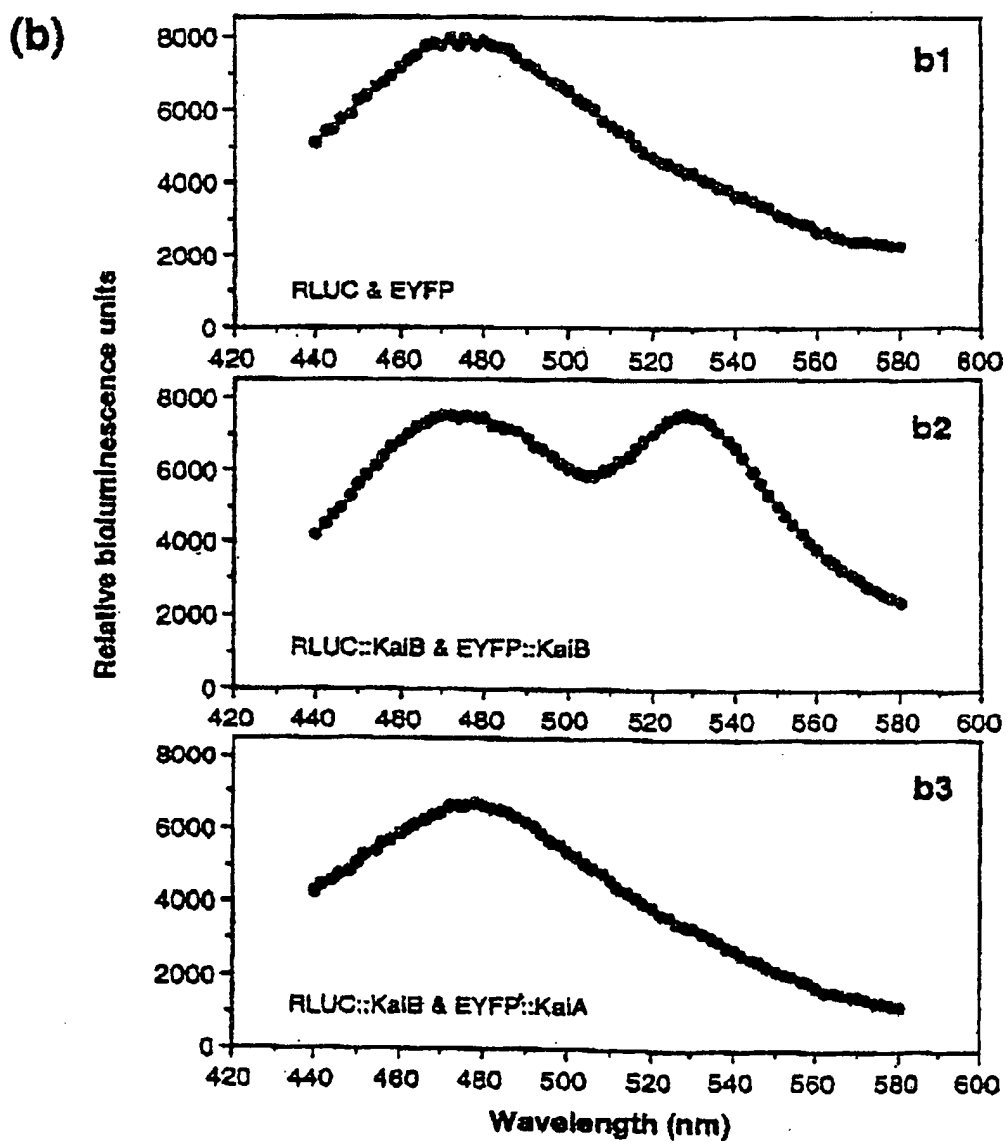


FIGURE 5

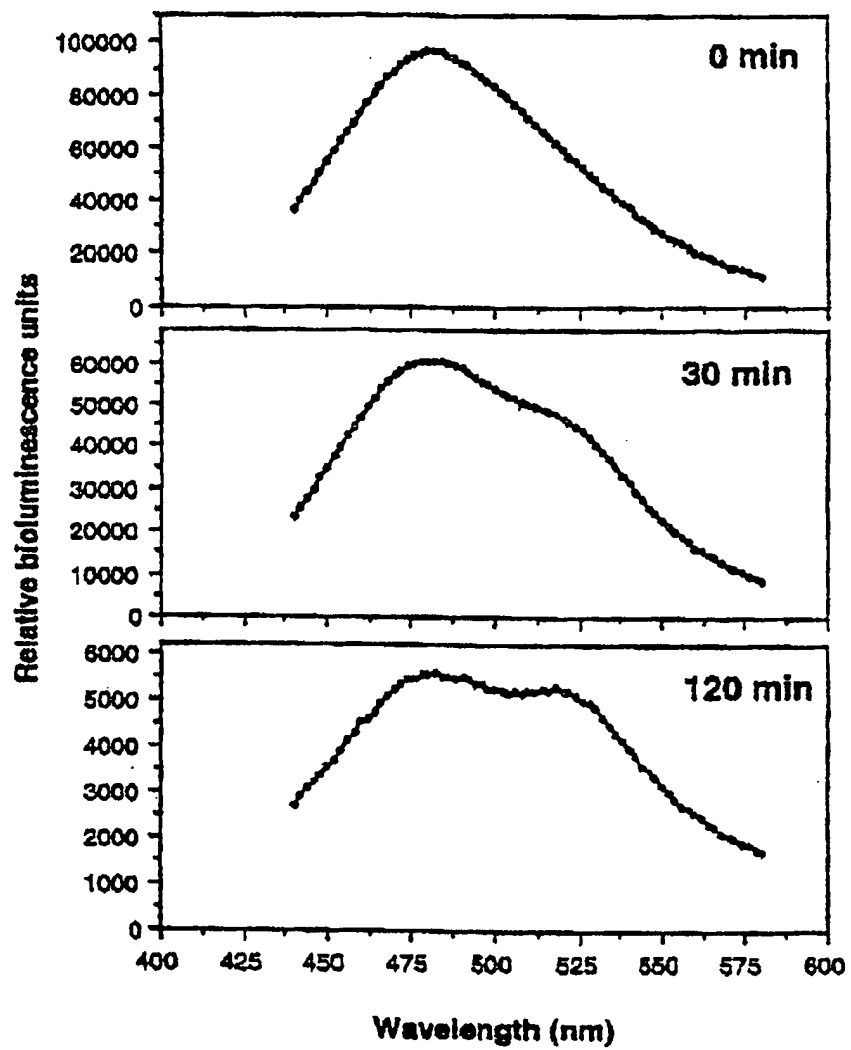


FIGURE 6

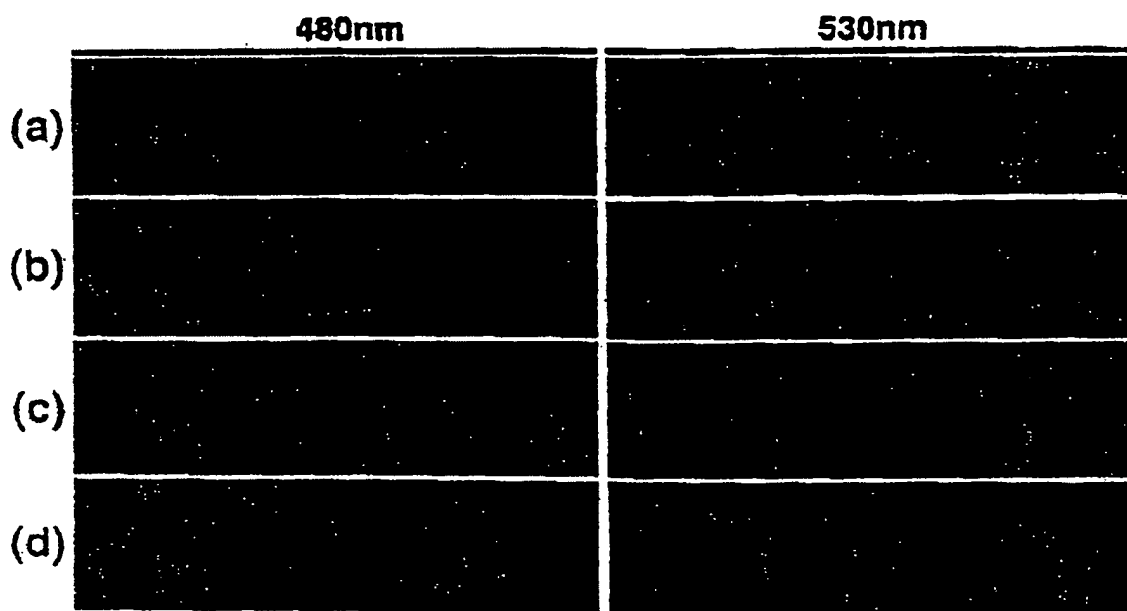


FIGURE 7

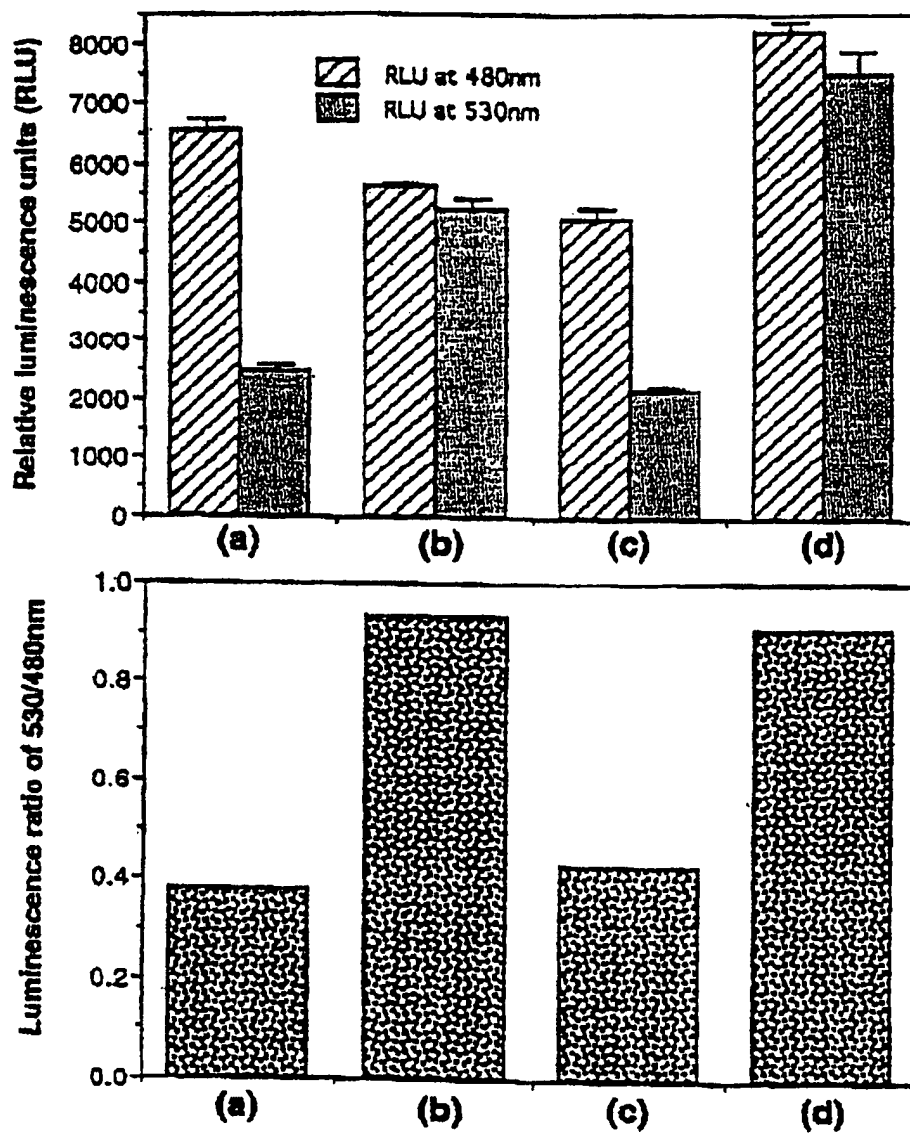


FIGURE 8

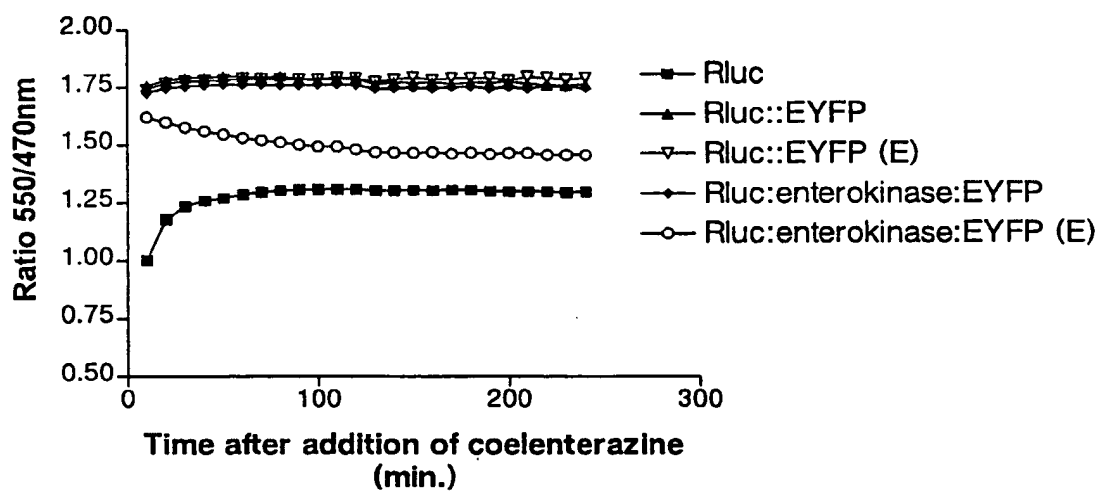
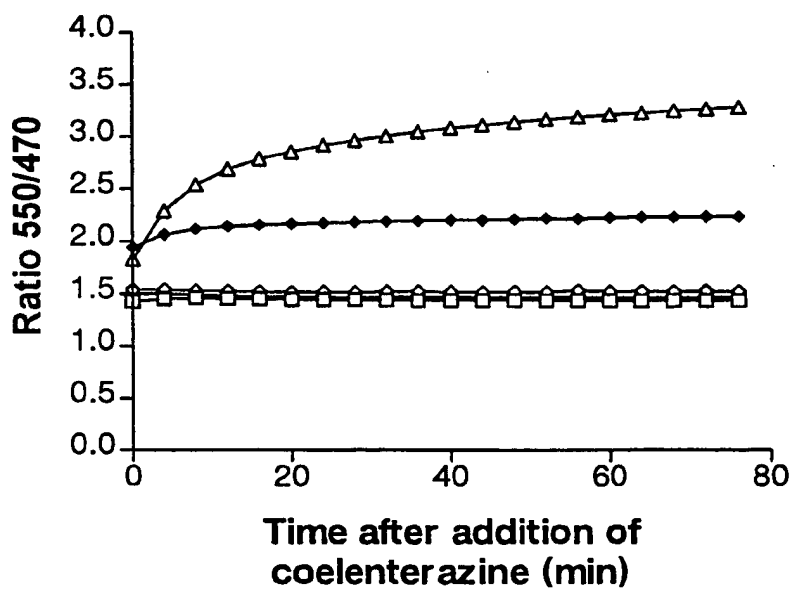


FIGURE 9



- Rluc-kaiB 4µg
- △— Rluc-KaiB + EYFPkaiB 4µg
- ◇— Rluc-kaiB + EYFPkaiA 4µg
- ▽— Rluc-kaiB 0.5µg
- Rluc-KaiB + EYFPkaiA 0.5µg
- +— Rluc-KaiB + EYFPkaiB 0.5µg



FIGURE 10

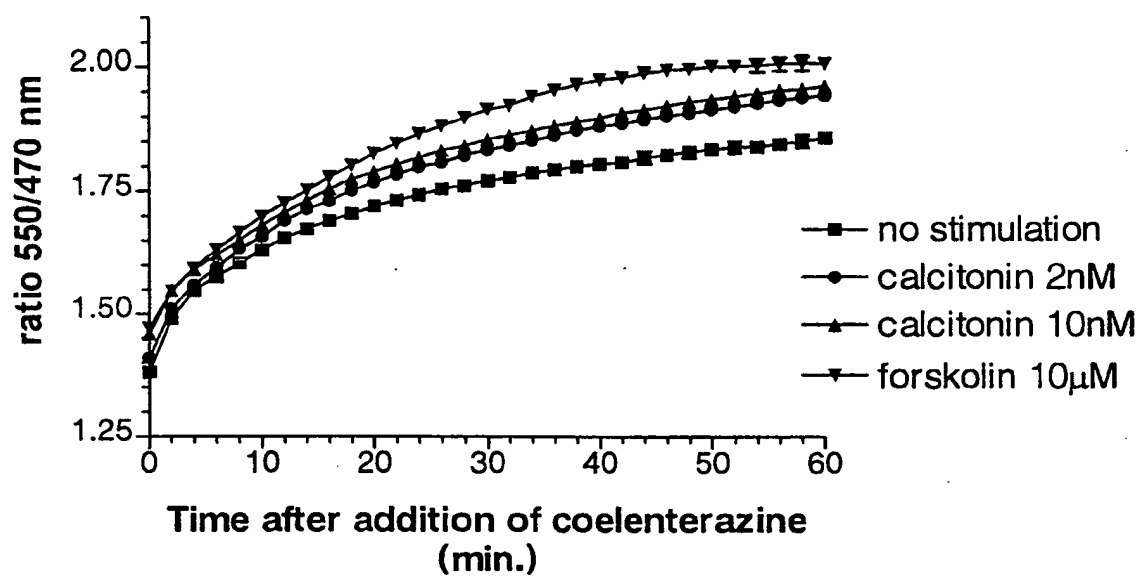


FIGURE 11

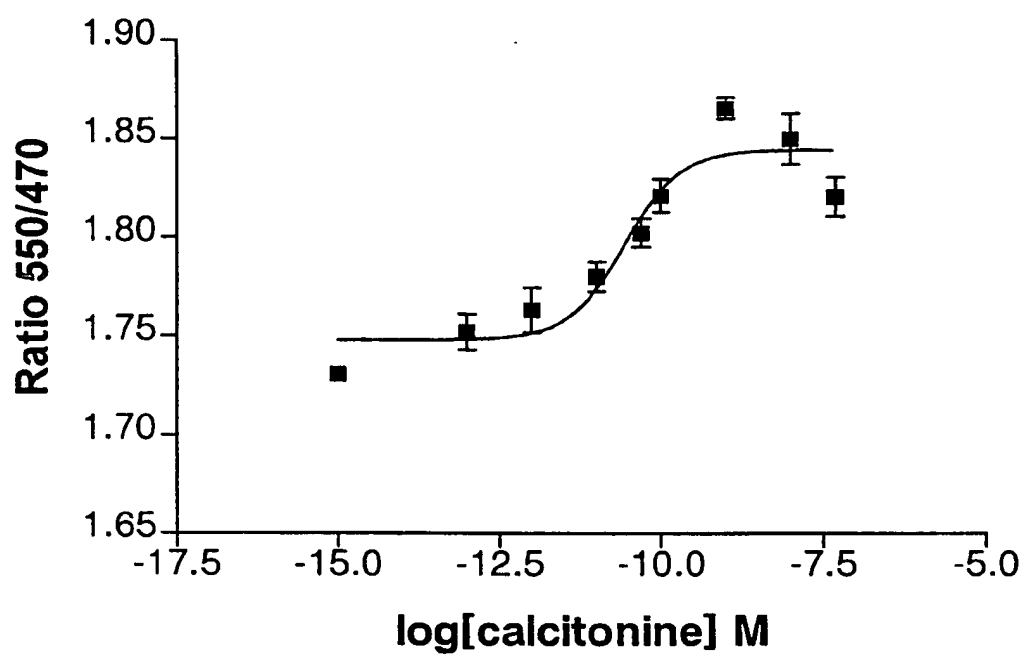


FIGURE 12

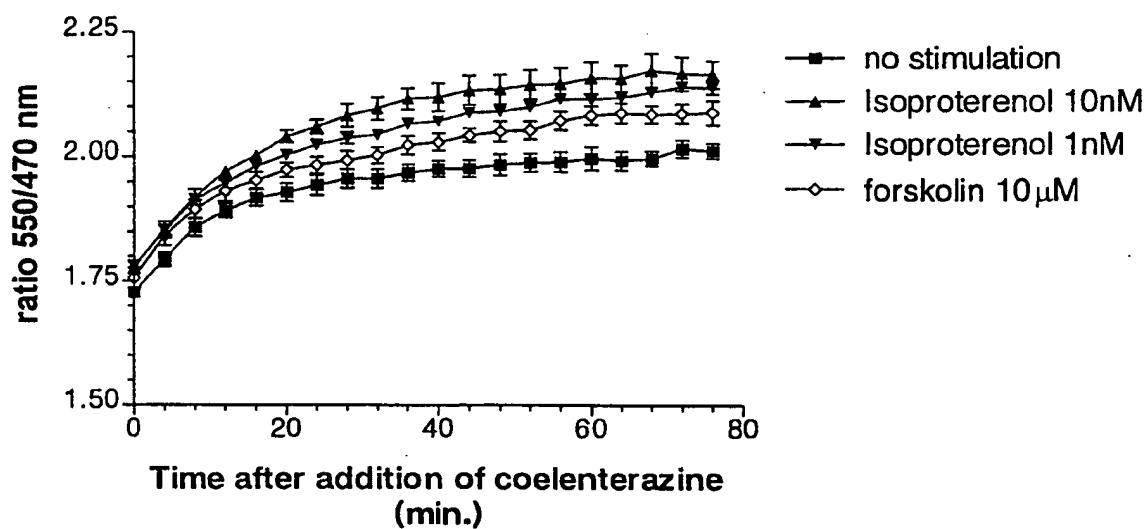


FIGURE 13

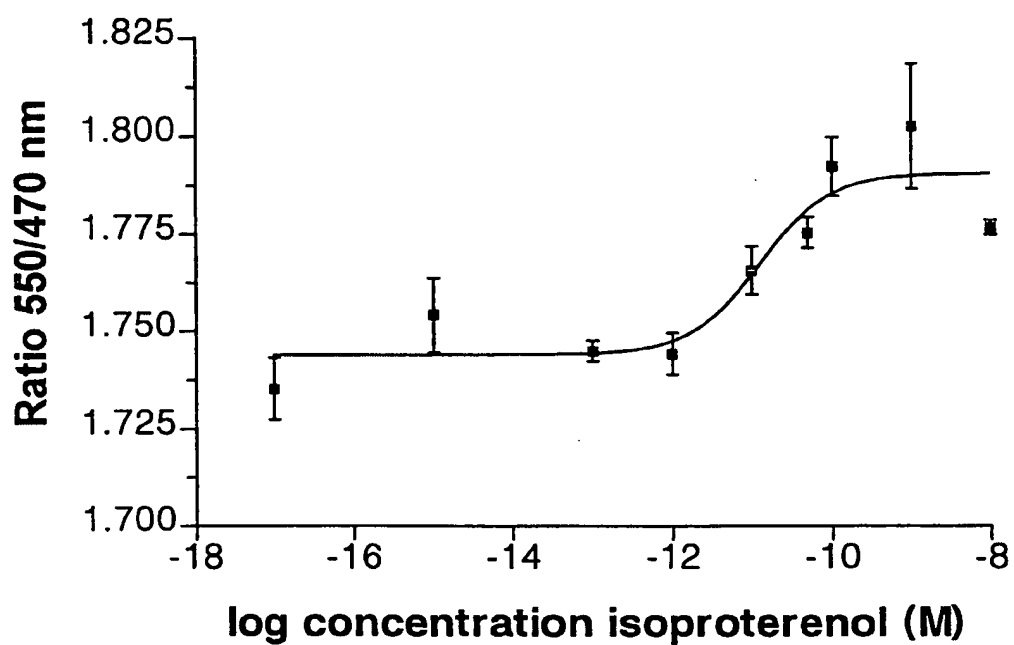


FIGURE 14

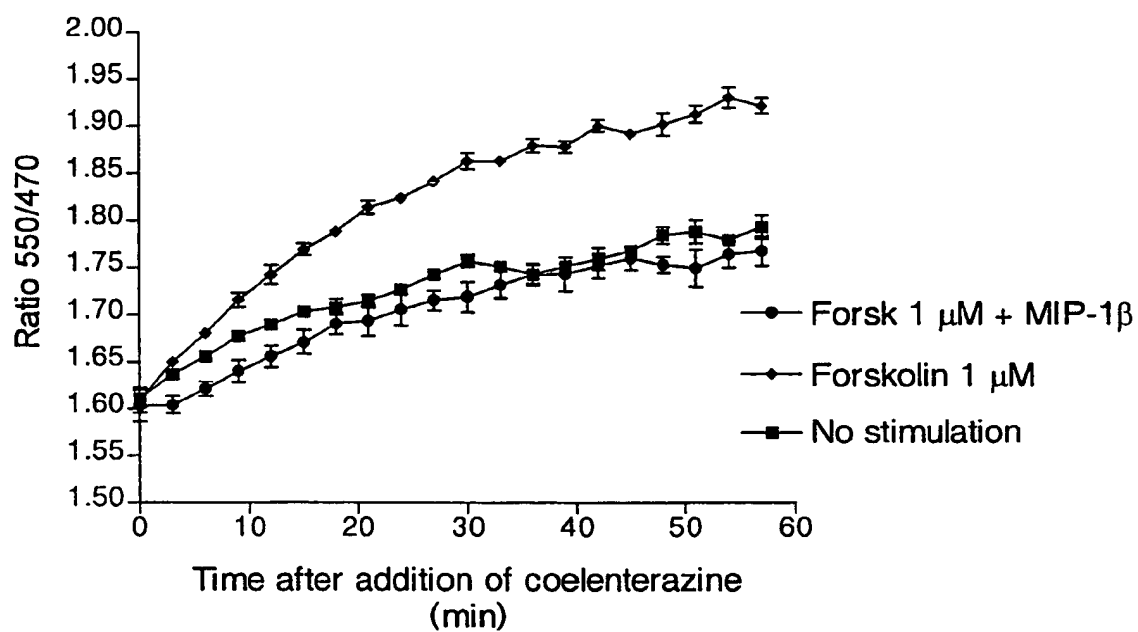


FIGURE 15

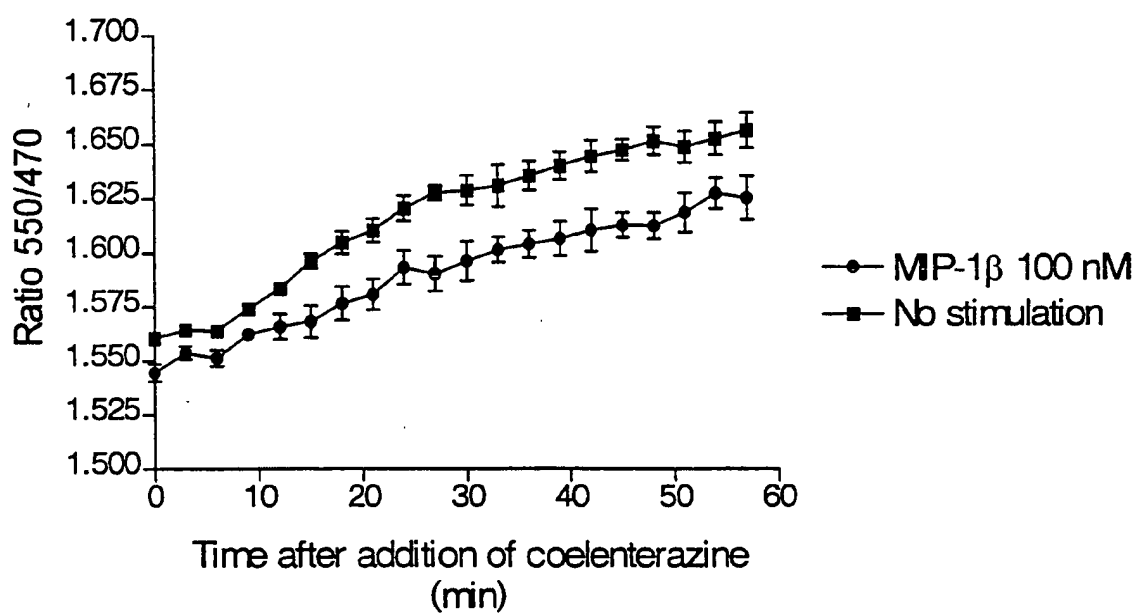


FIGURE 16

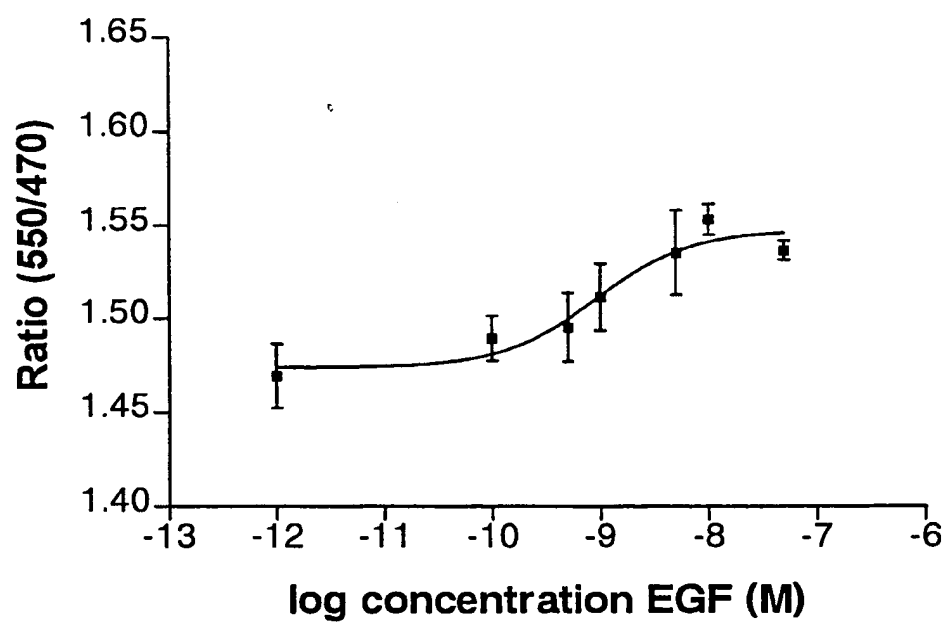


FIGURE 17

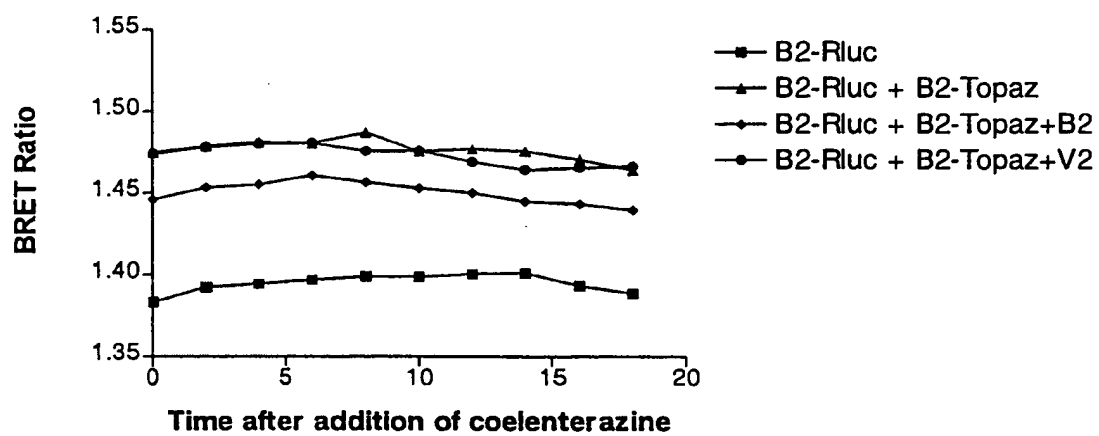




FIGURE 18

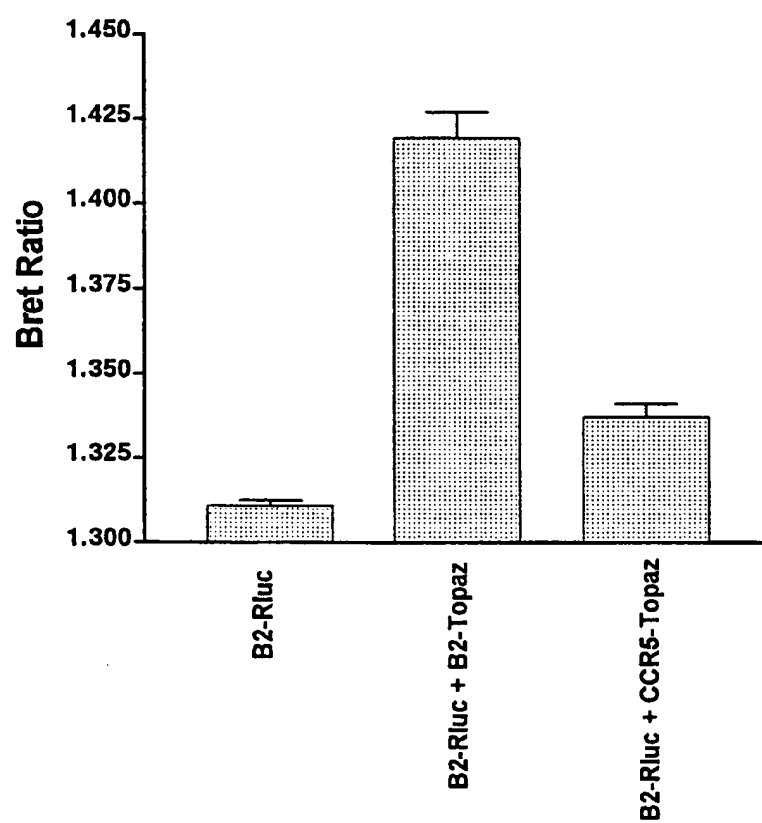


FIGURE 19

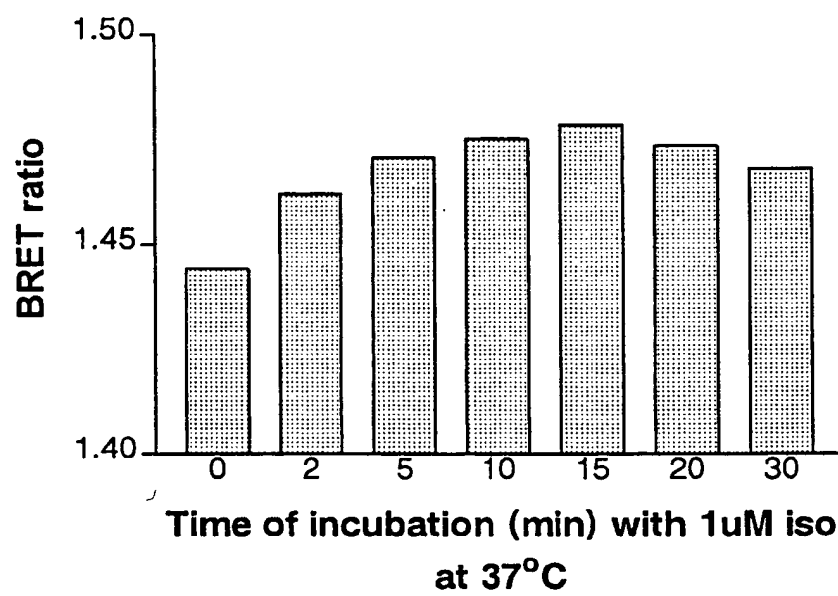


FIGURE 20

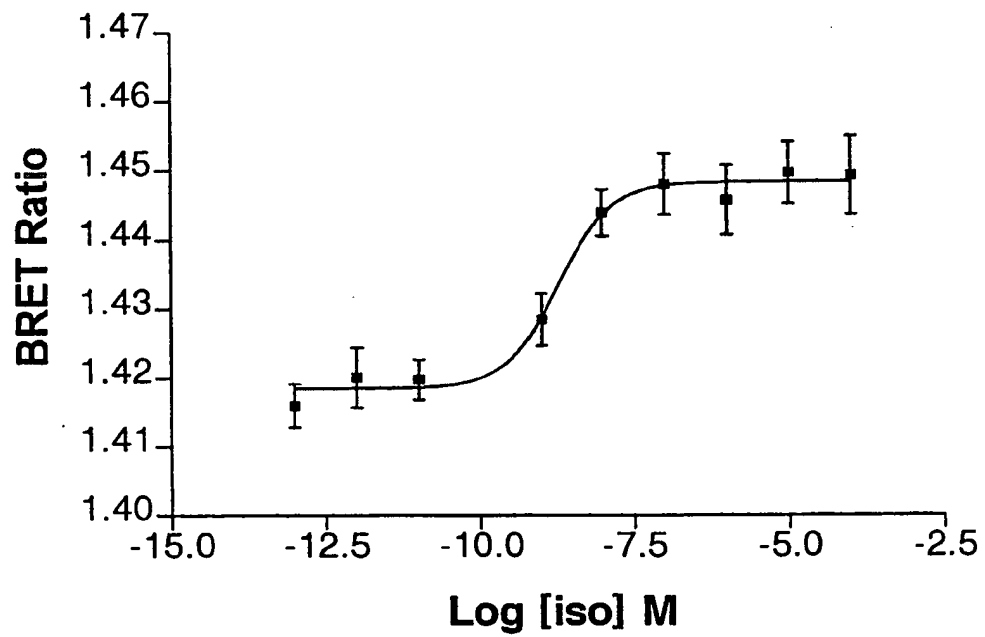


FIGURE 21

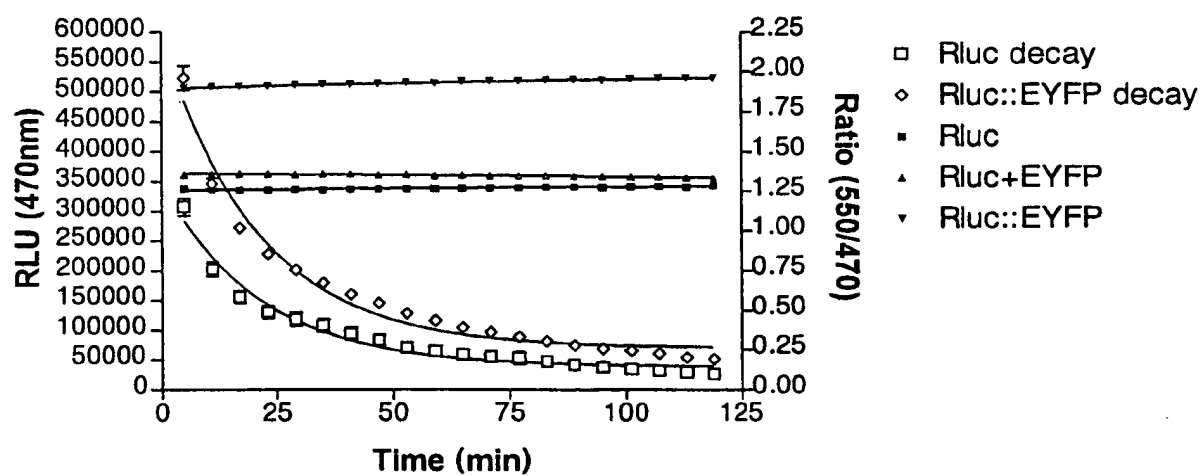
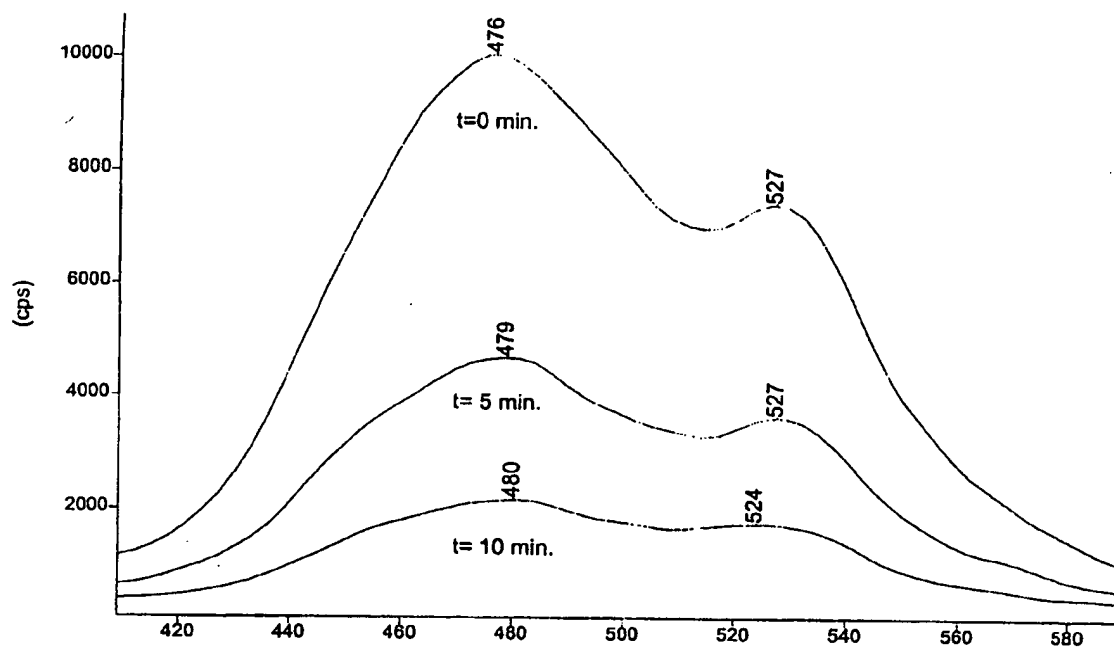


FIGURE 22



Emission Acquisition

FIGURE 23

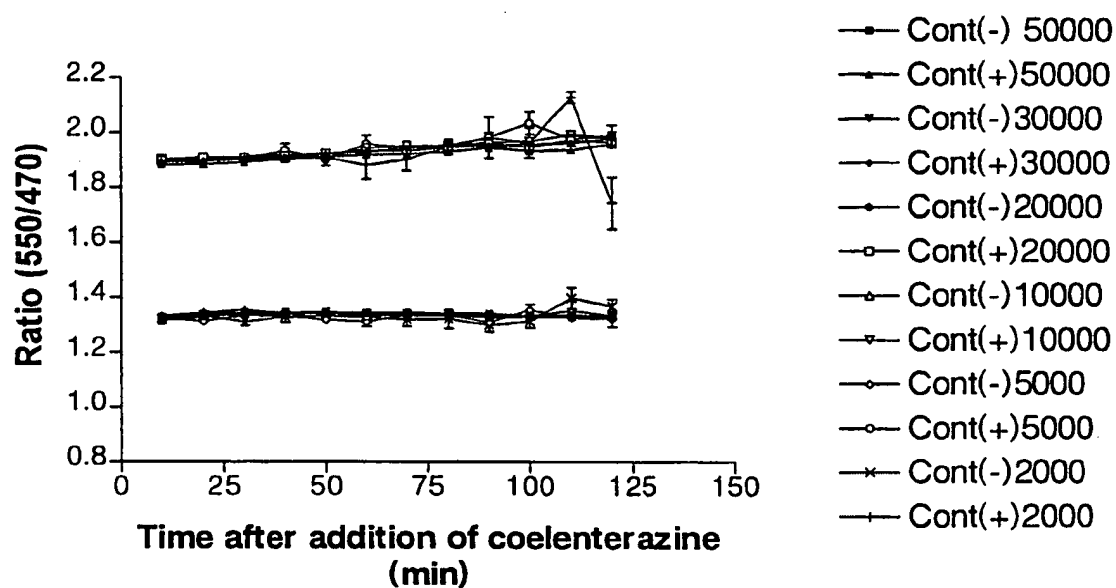


FIGURE 24

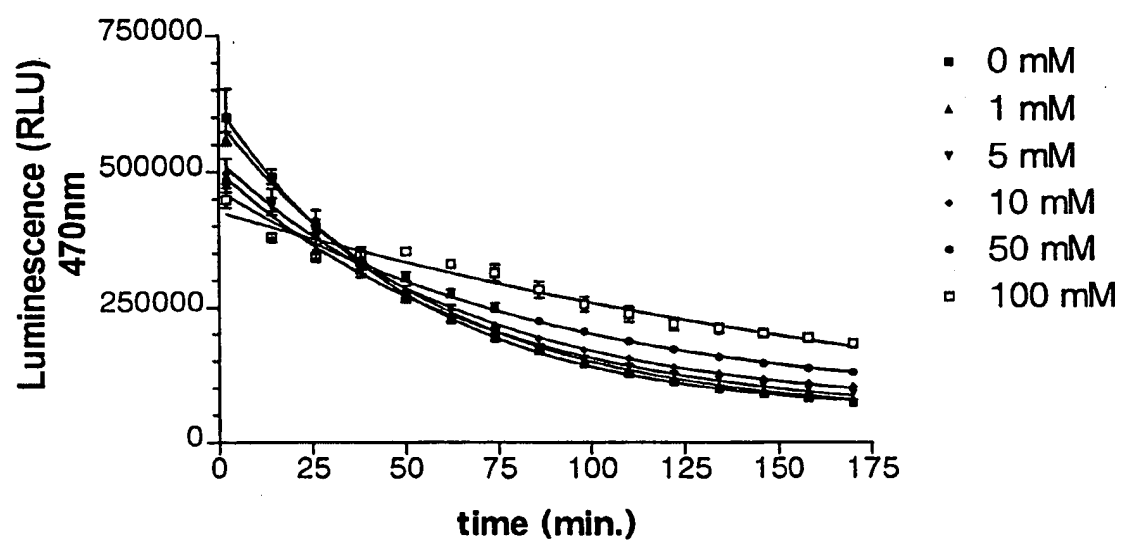


FIGURE 25

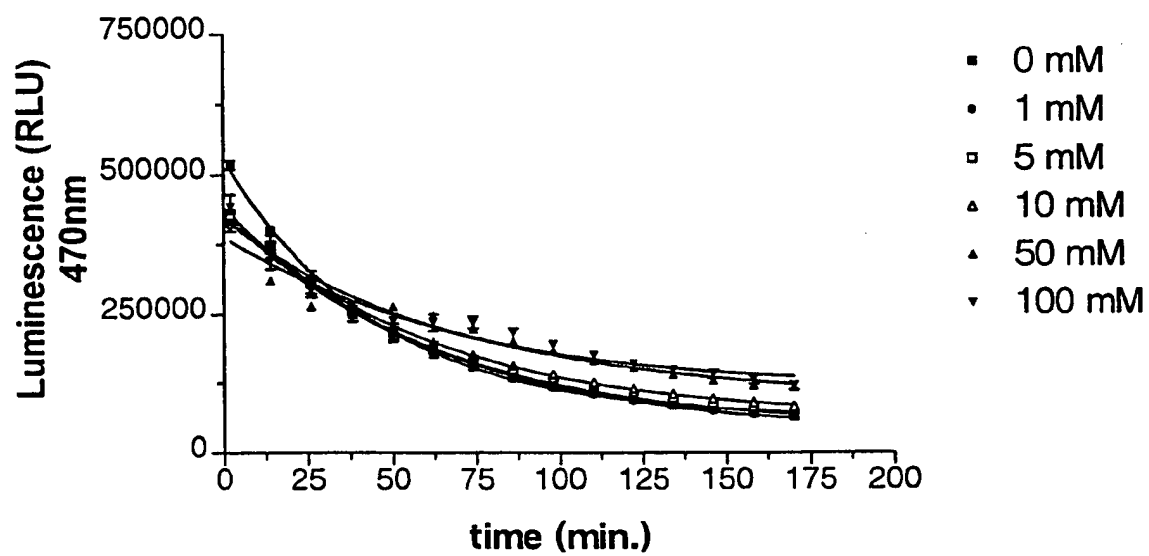




FIGURE 26

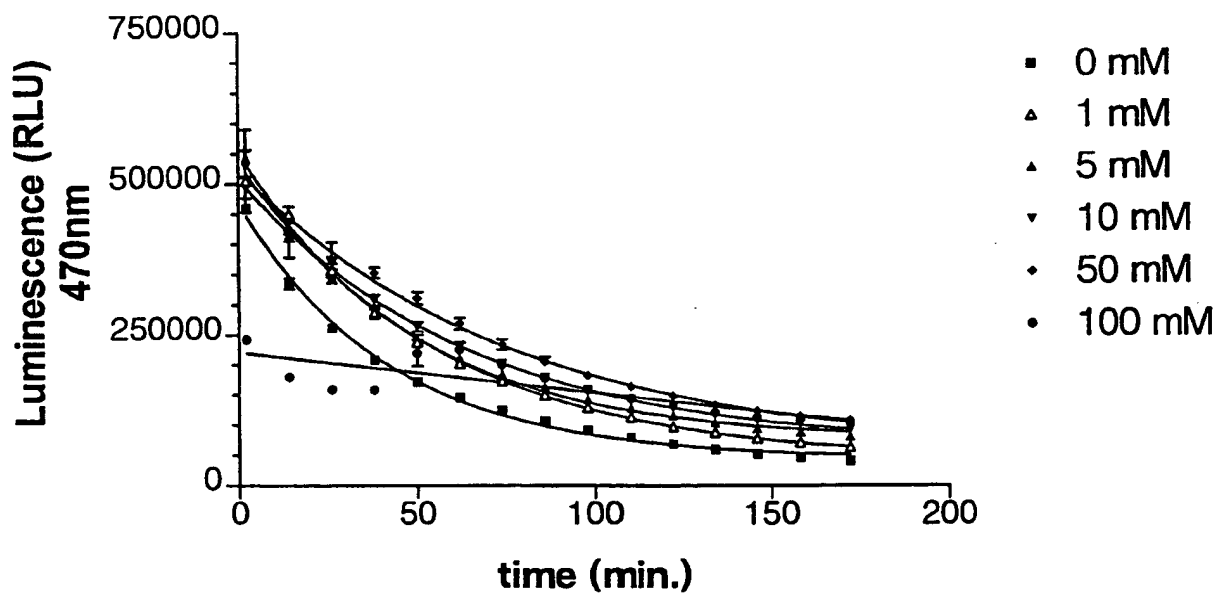


FIGURE 27

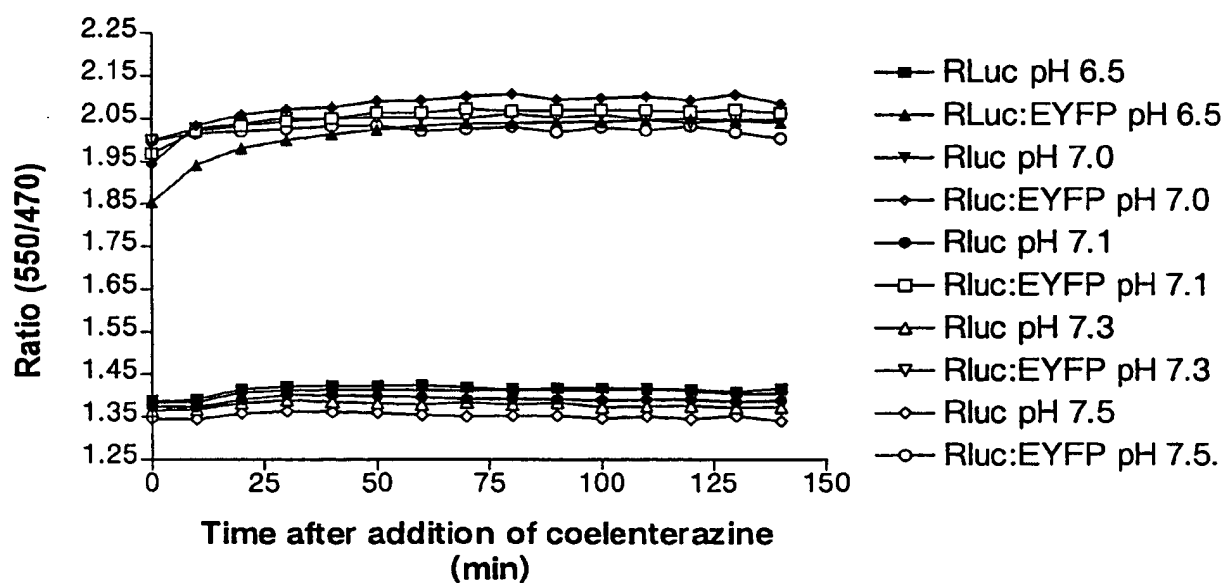


FIGURE 28

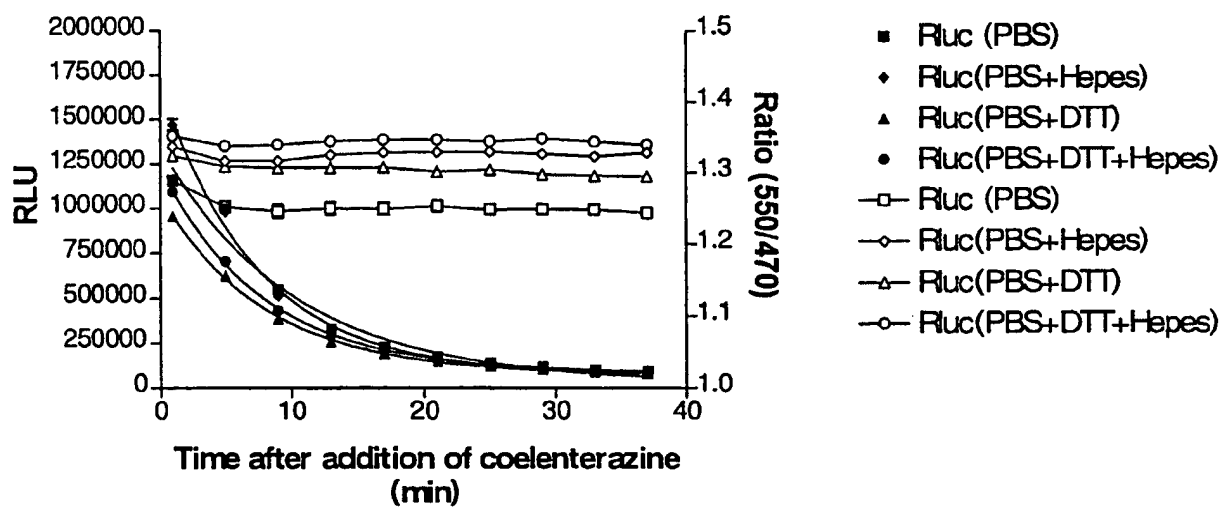


FIGURE 29

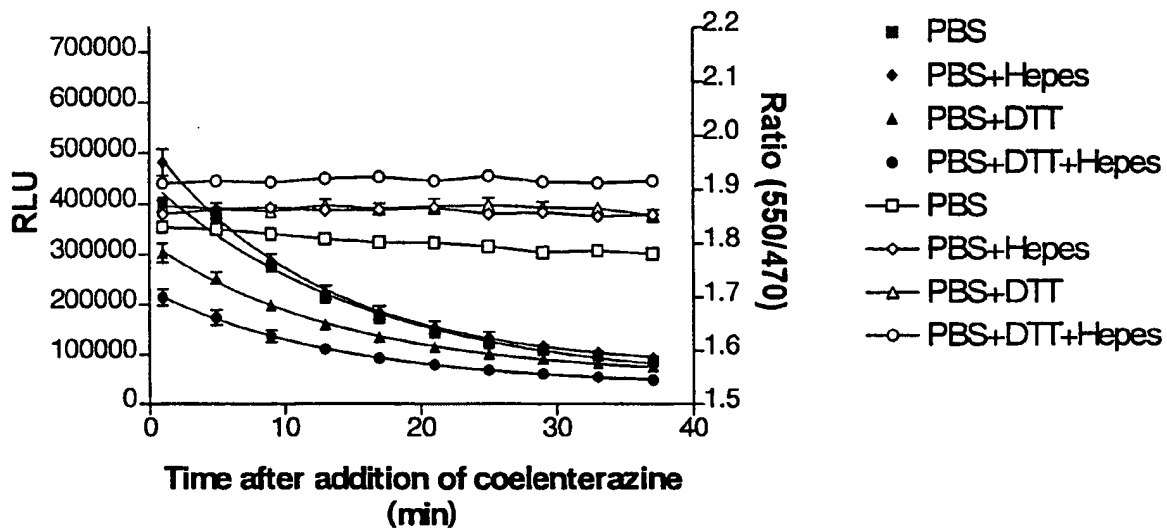


FIGURE 30

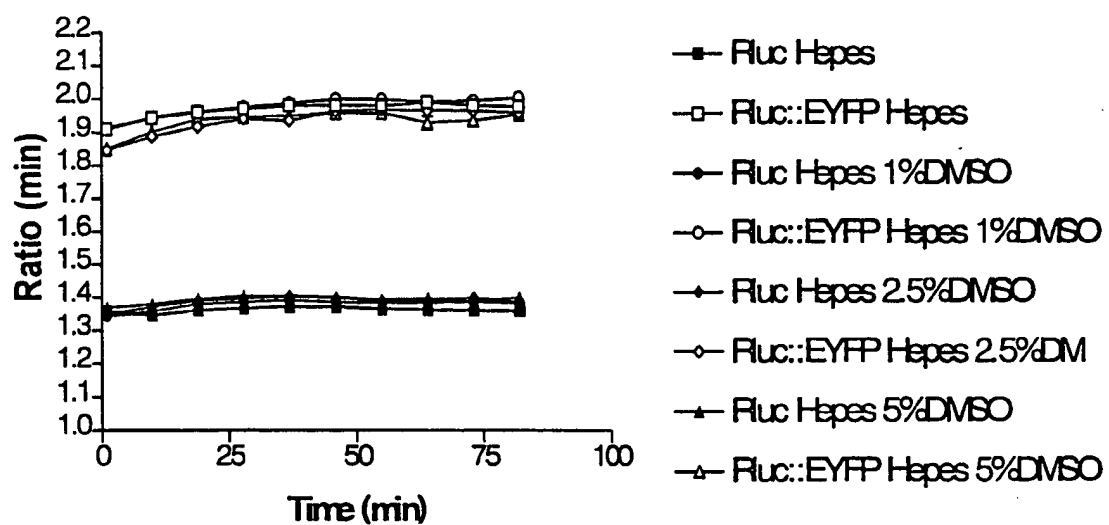


FIGURE 31

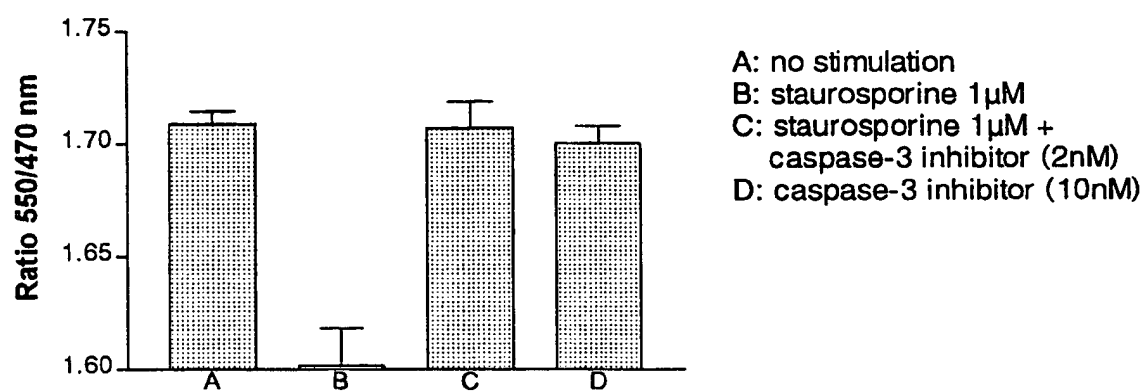


FIGURE 32

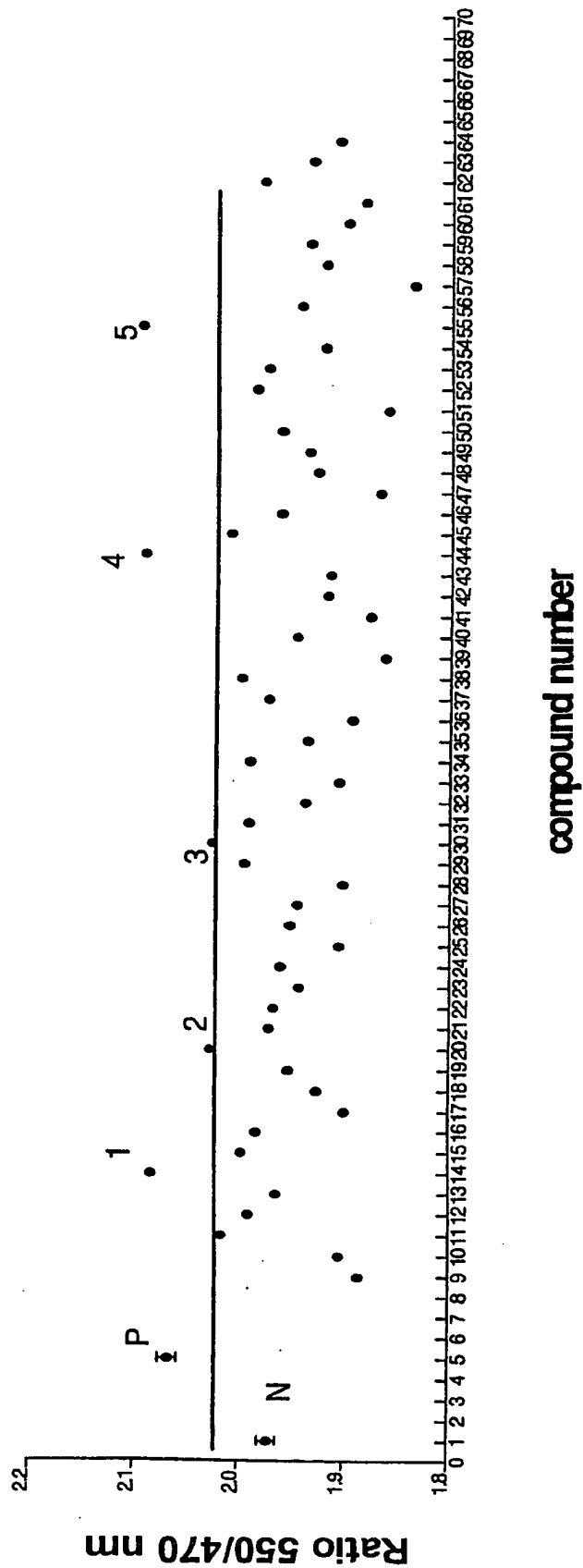


FIGURE 33

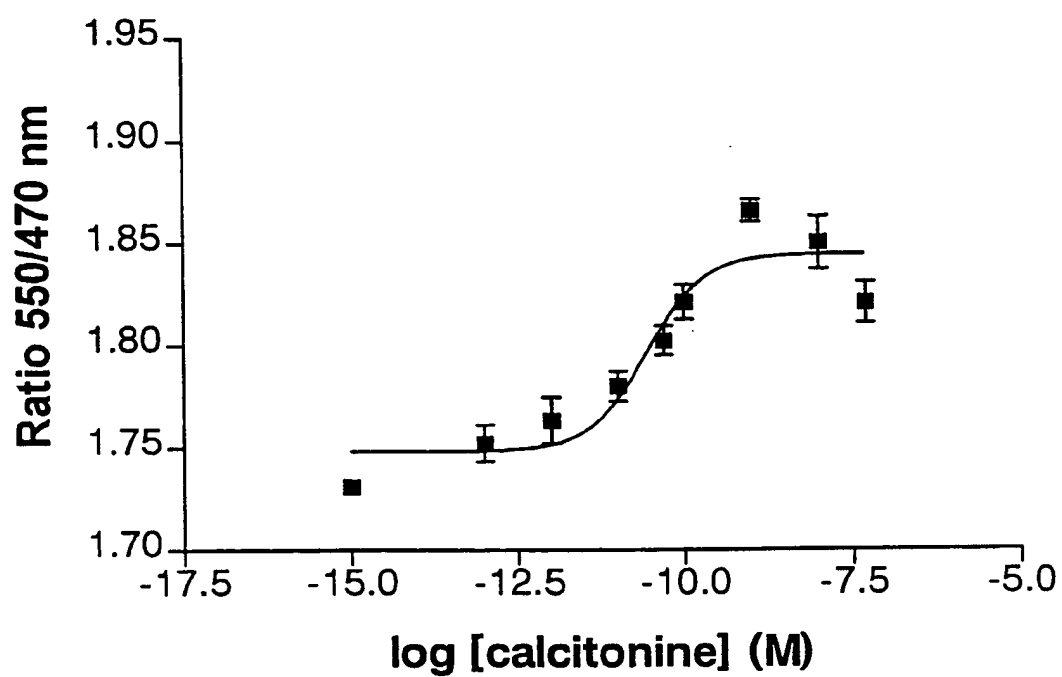




FIGURE 34

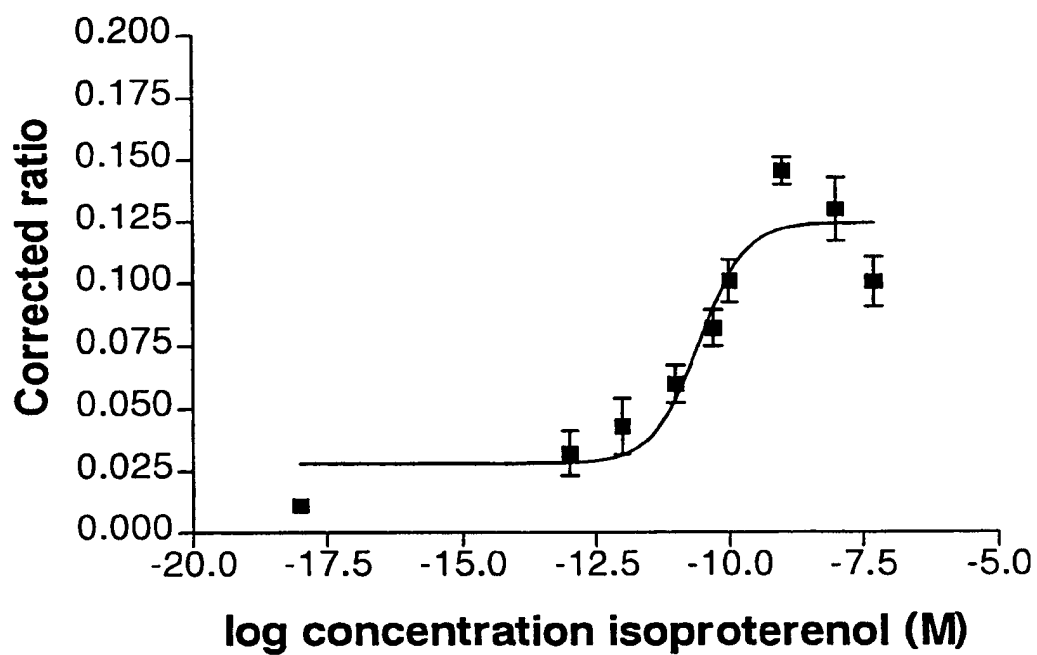


FIGURE 35

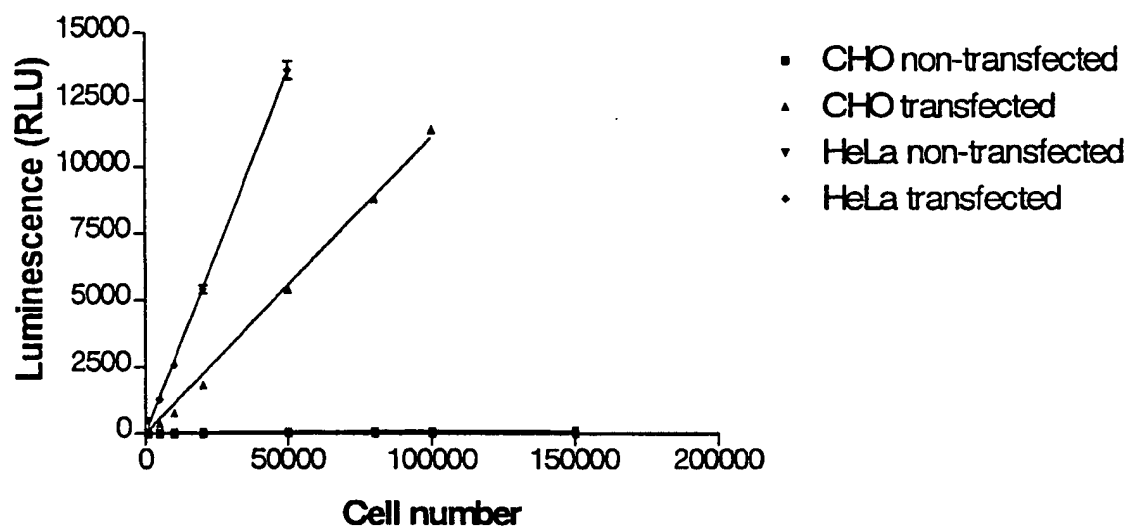


FIGURE 36

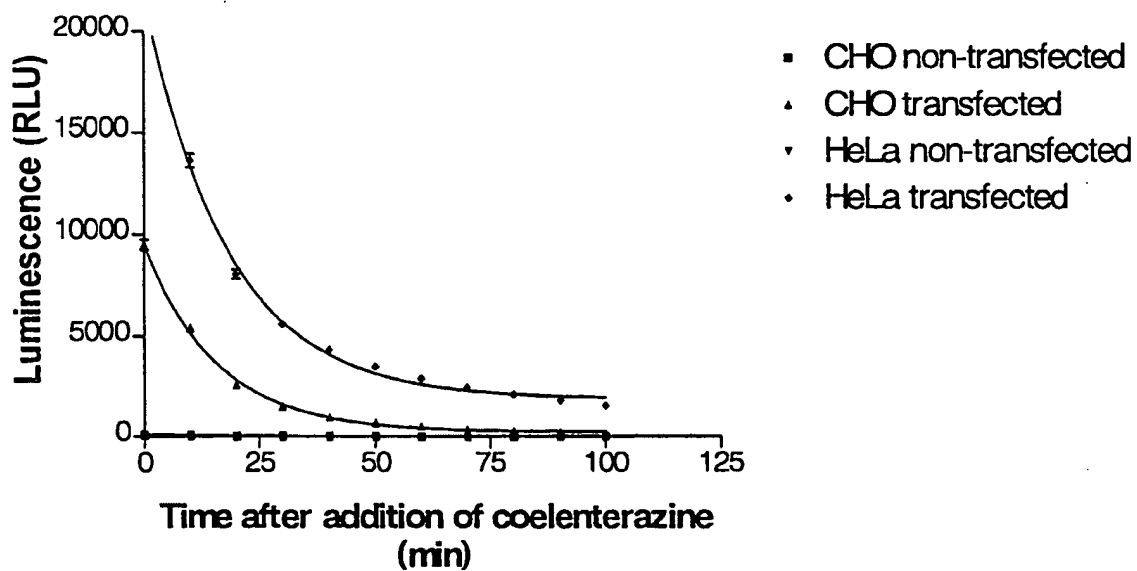


FIGURE 37

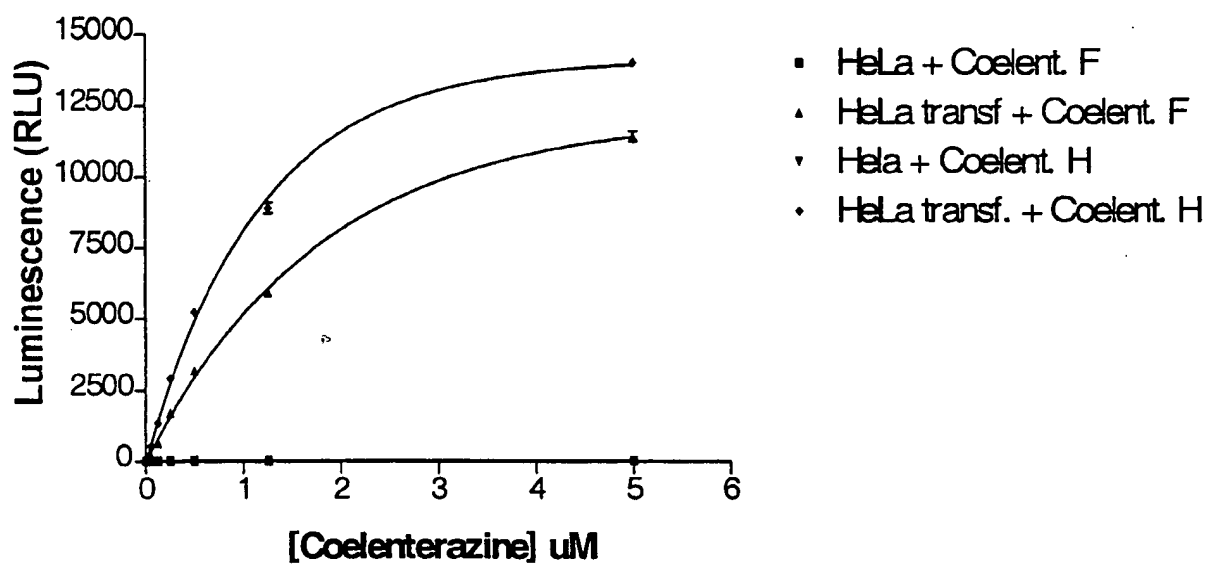


FIGURE 38

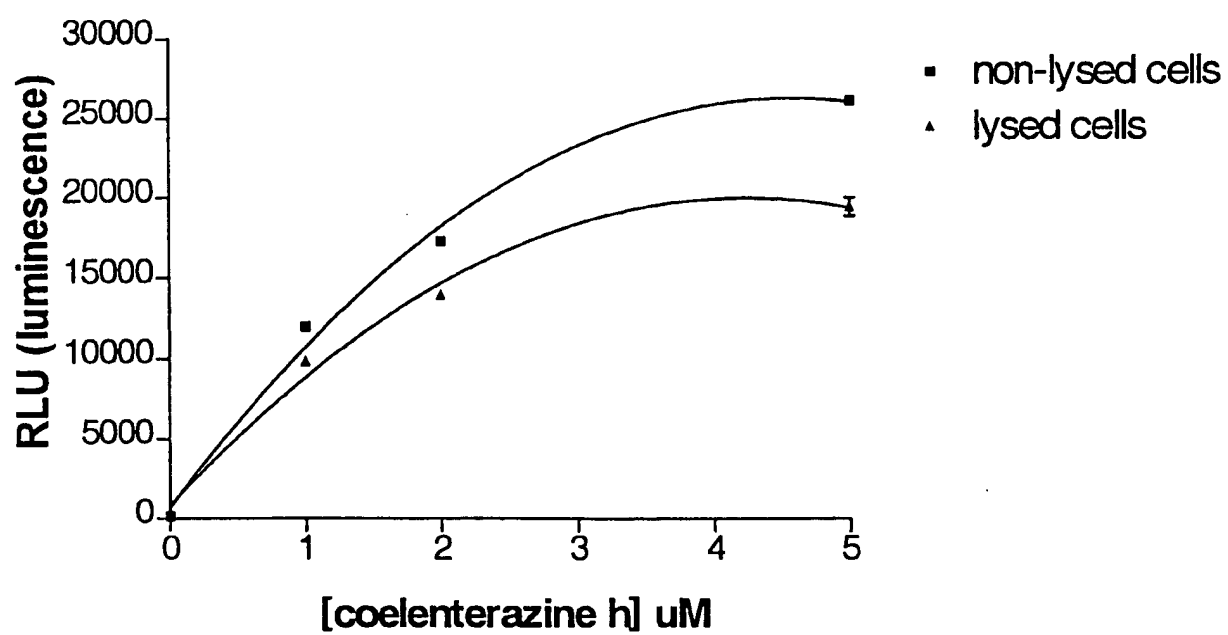


FIGURE 39

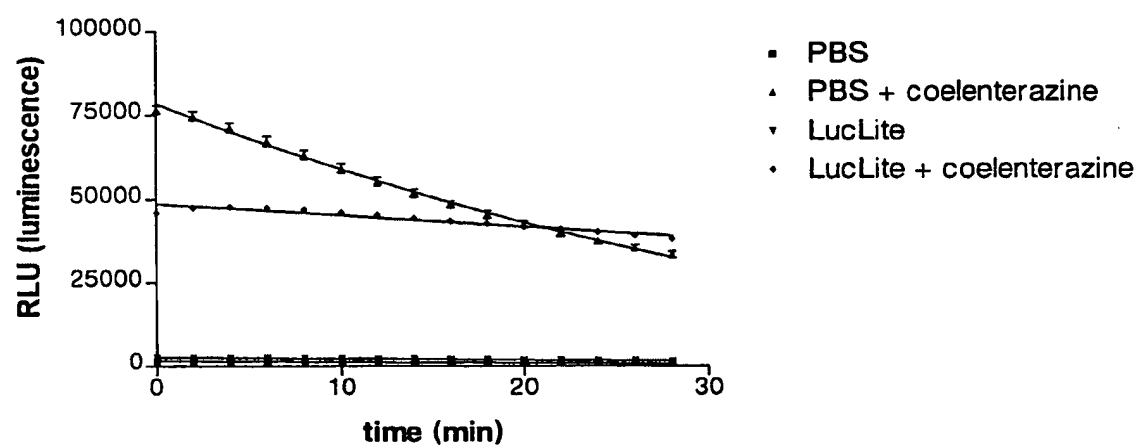


FIGURE 40

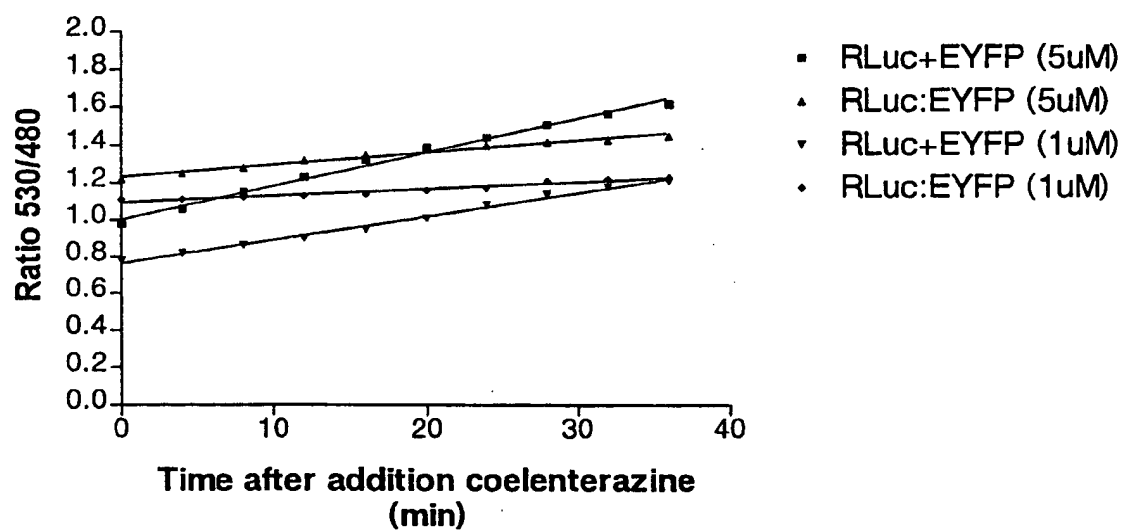


FIGURE 41

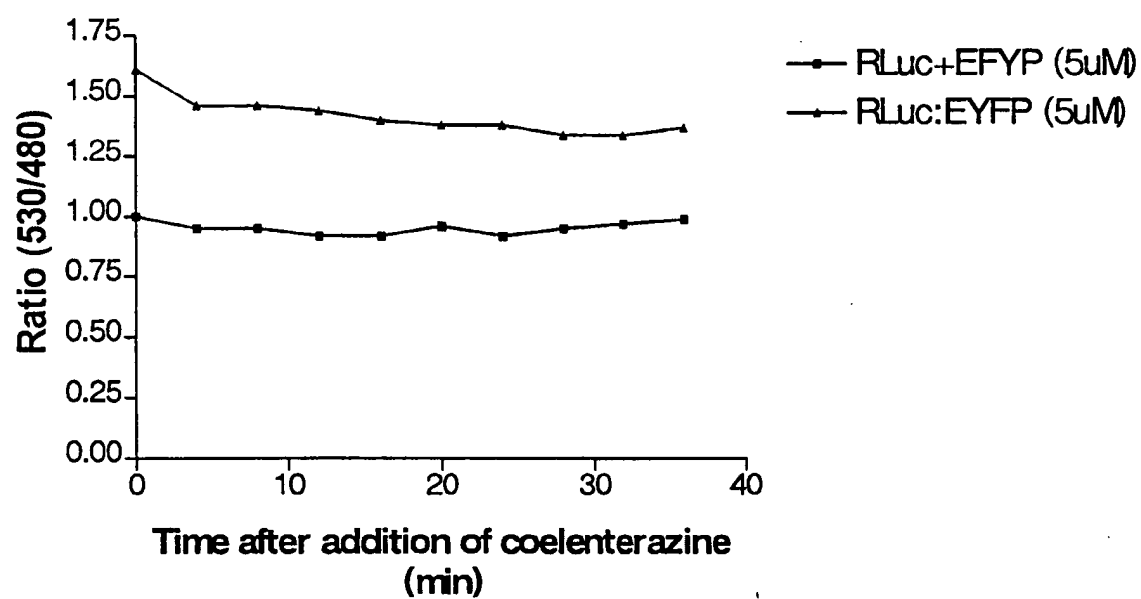




FIGURE 42

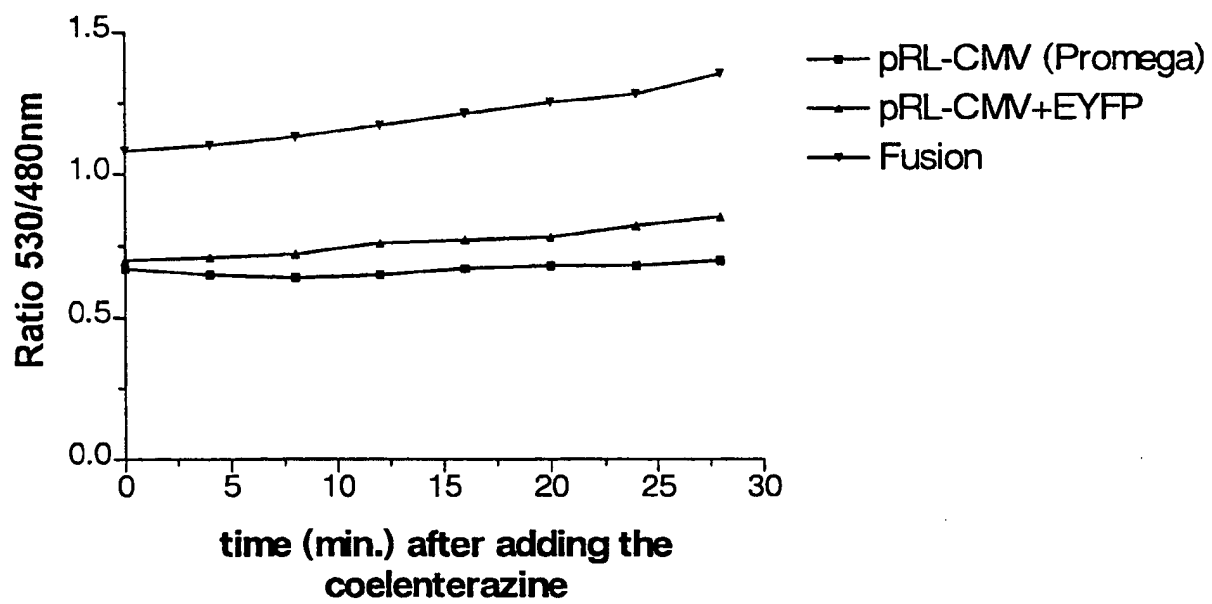


FIGURE 43

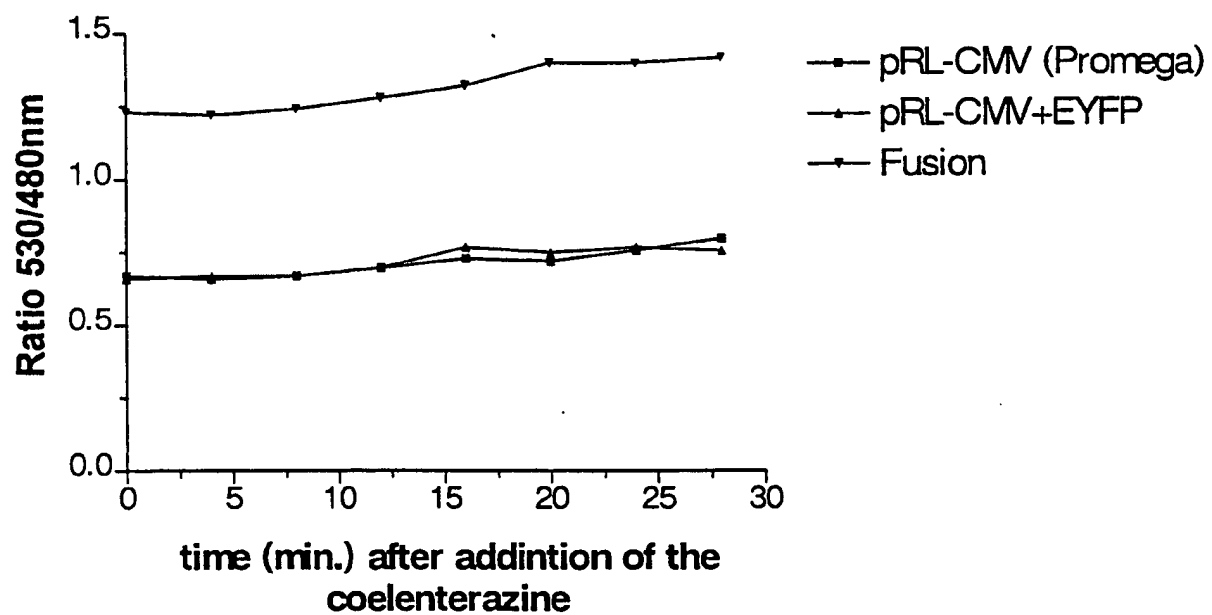


FIGURE 44

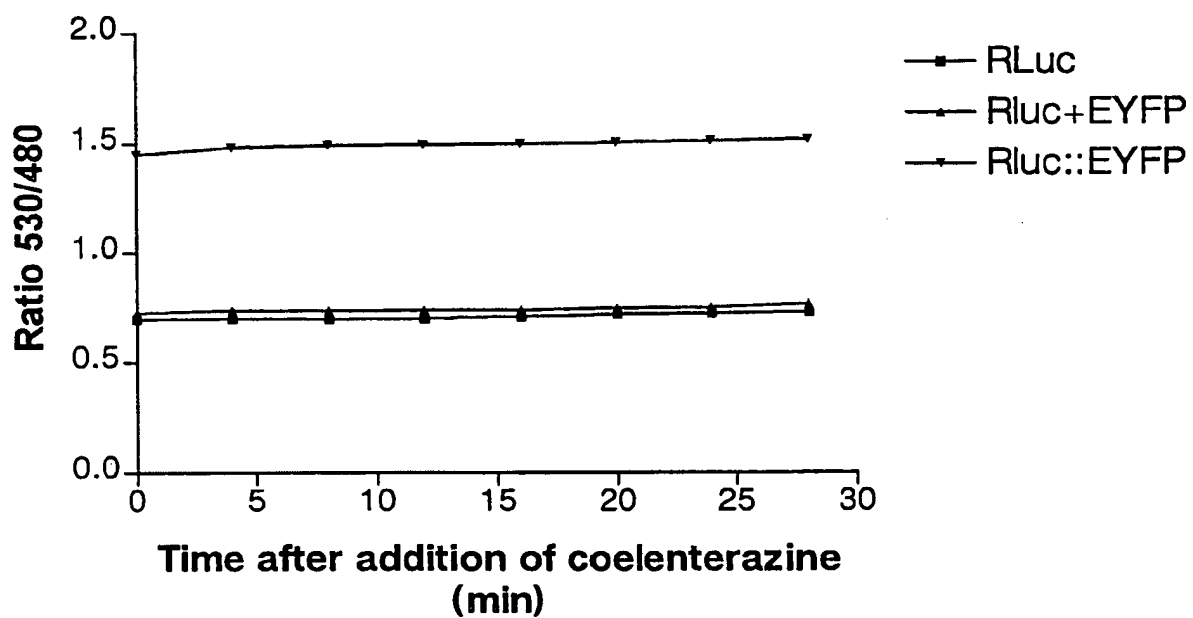


FIGURE 45

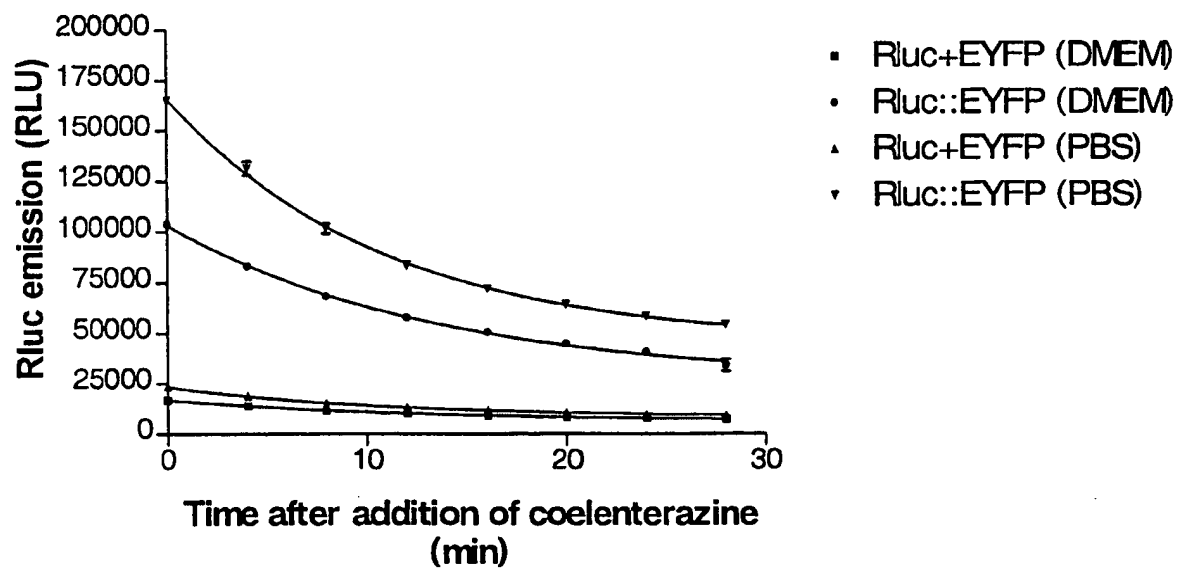


FIGURE 46

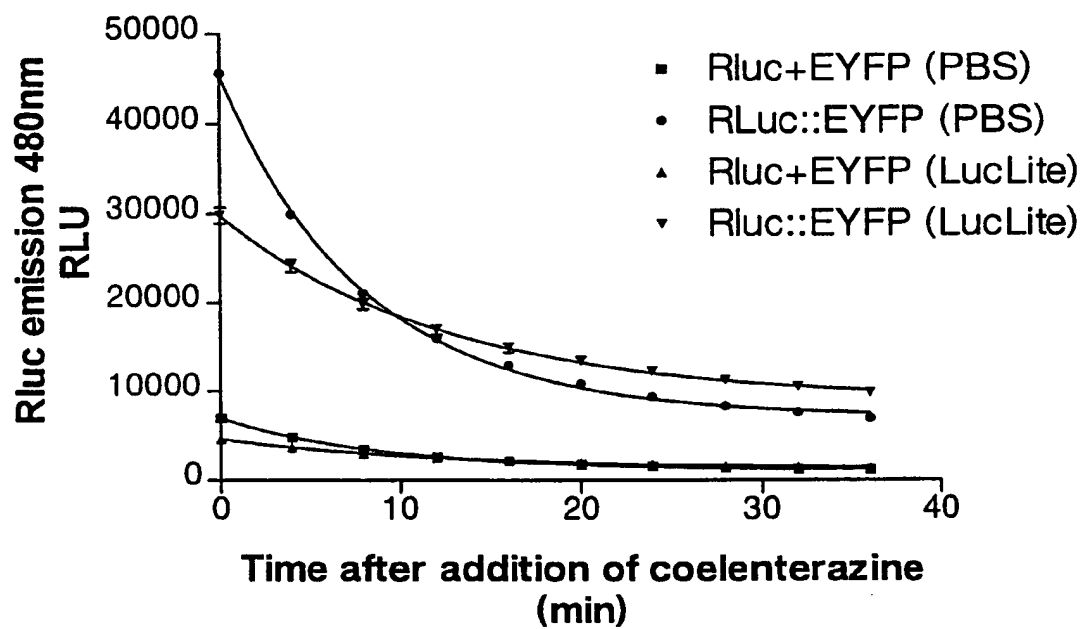


FIGURE 47

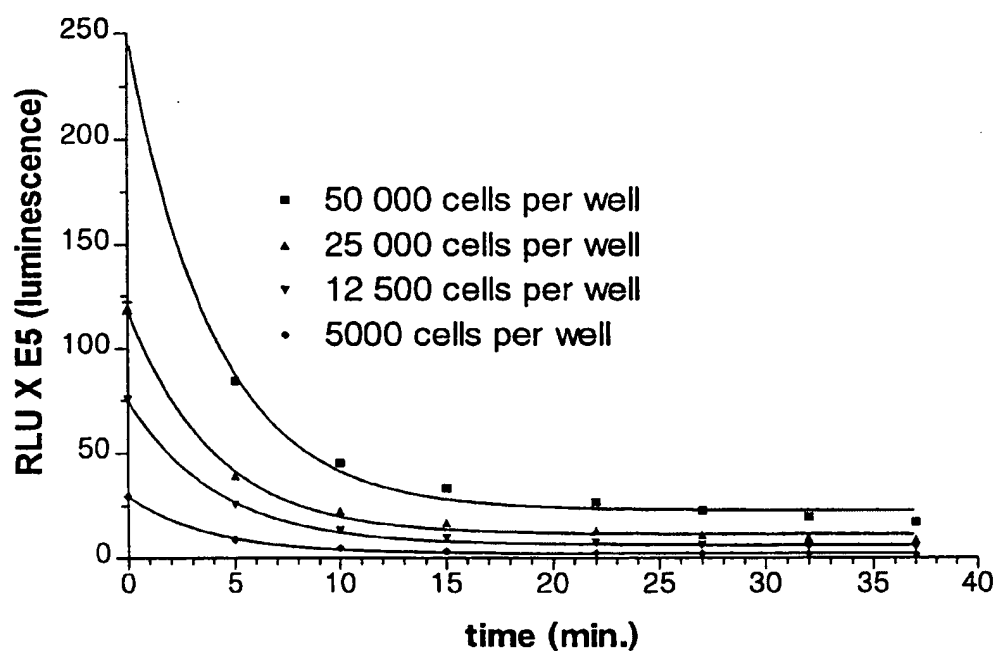


FIGURE 48

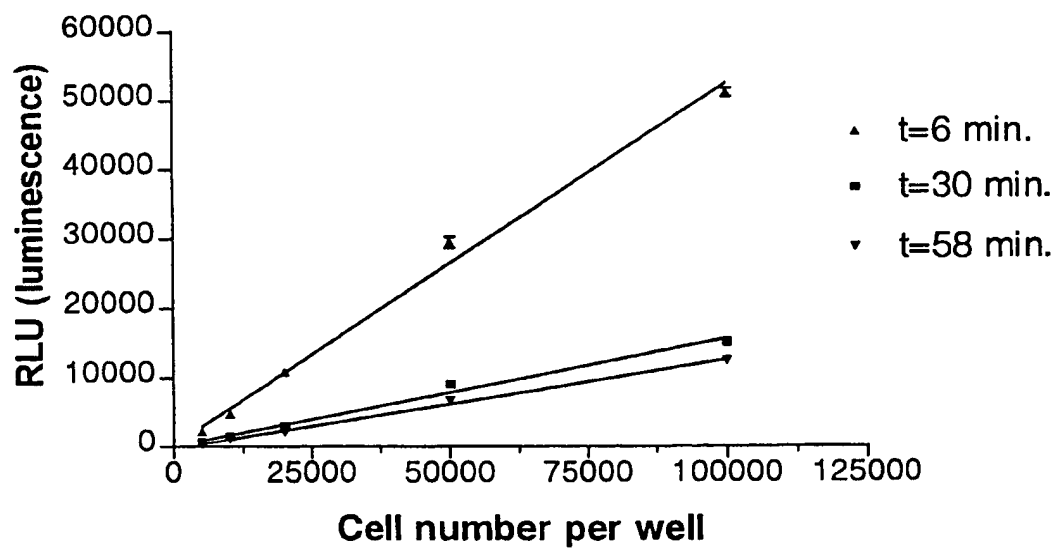


FIGURE 49

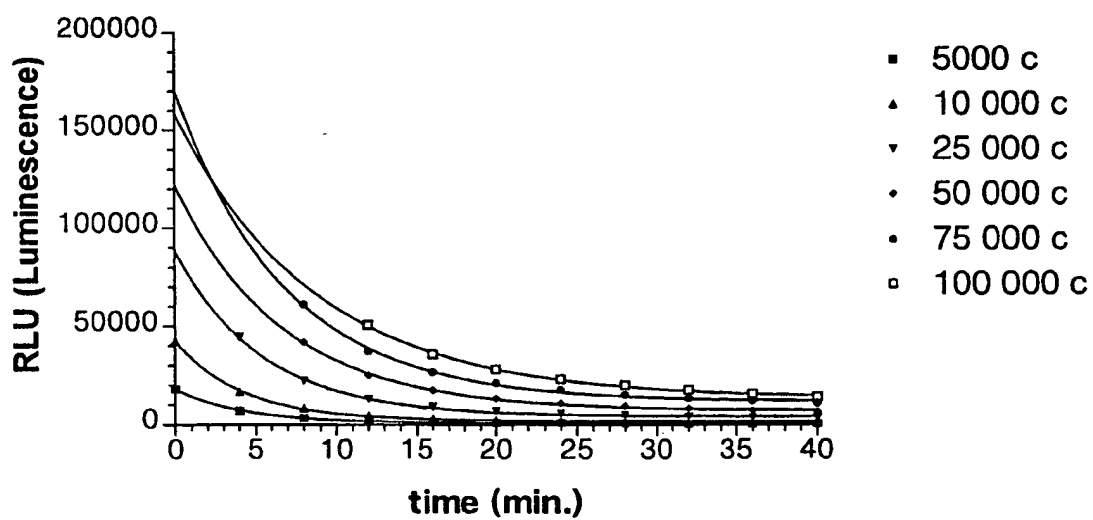




FIGURE 50

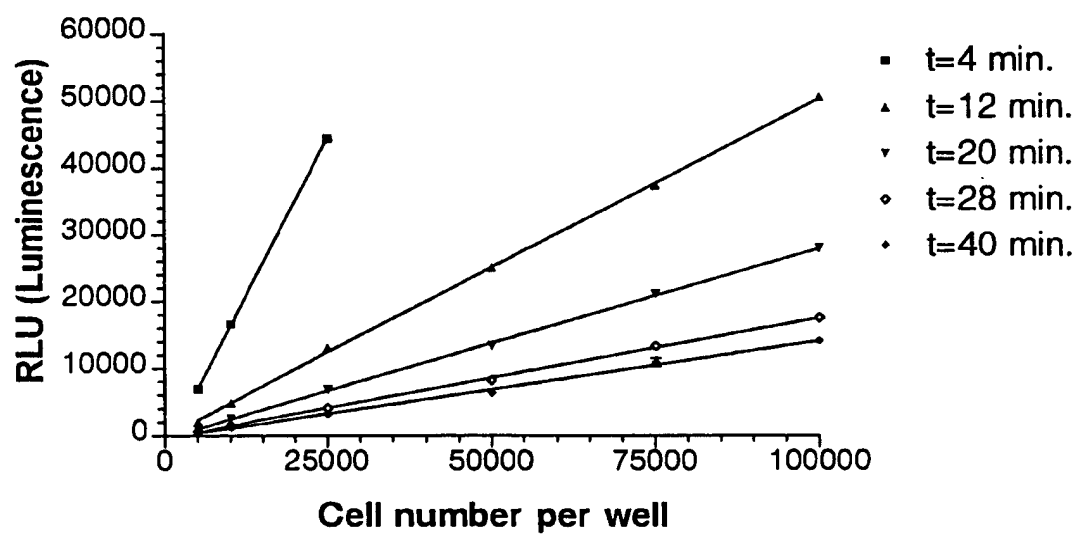


FIGURE 51

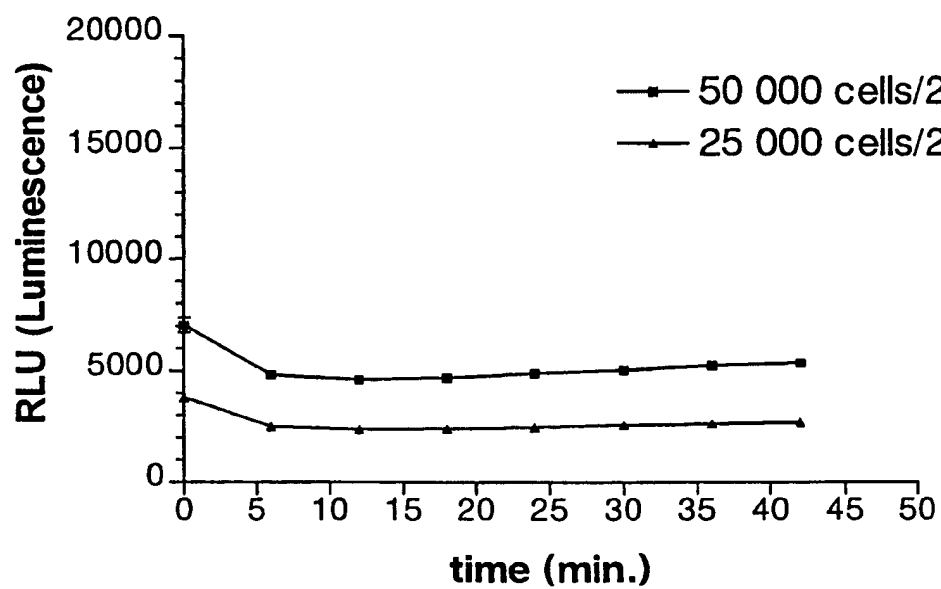


FIGURE 52

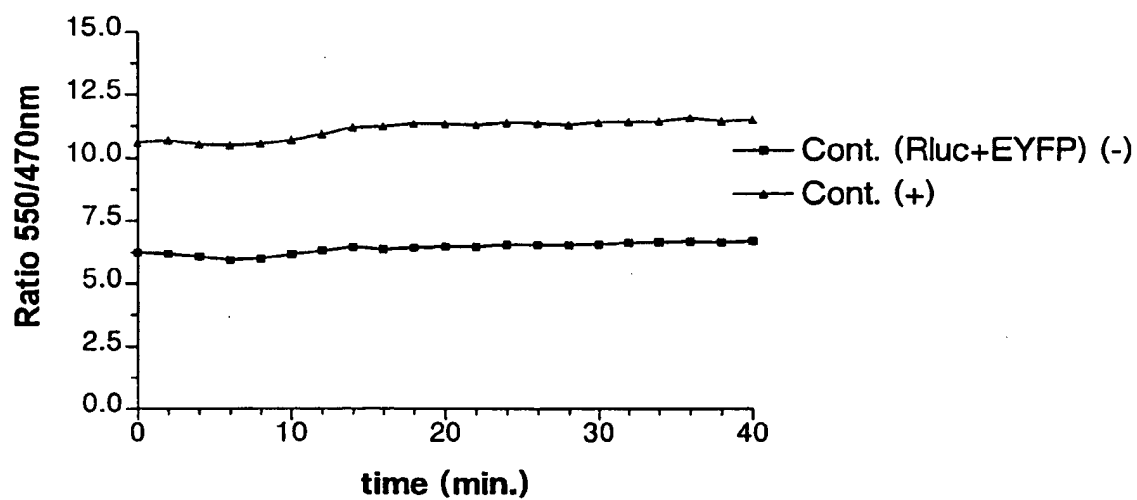


FIGURE 53

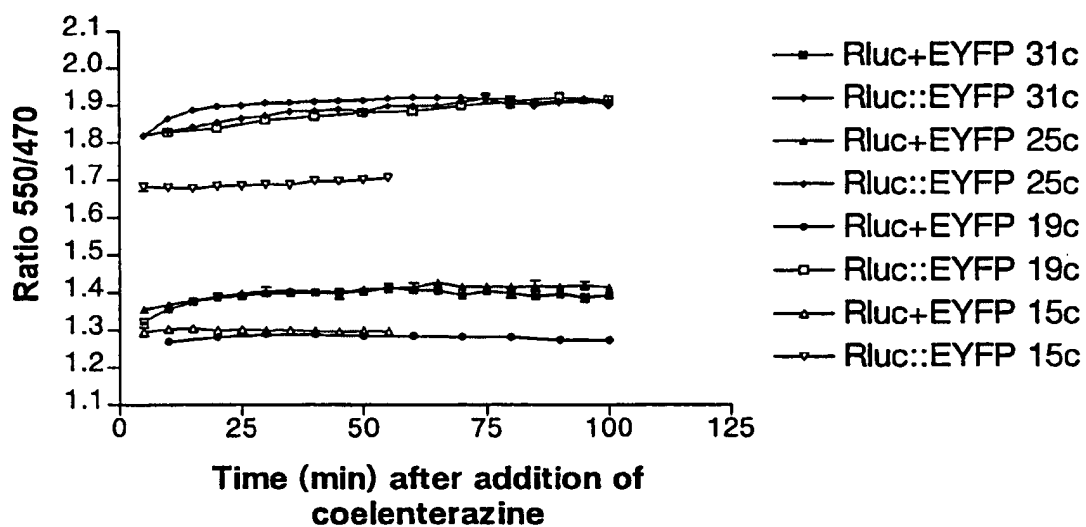


FIGURE 54

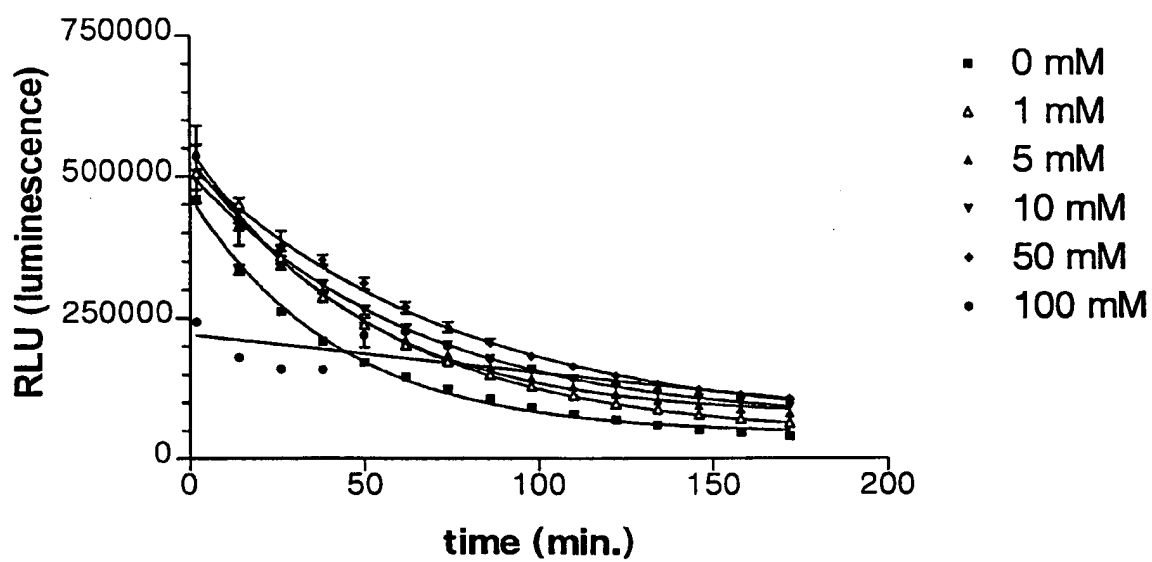


FIGURE 55

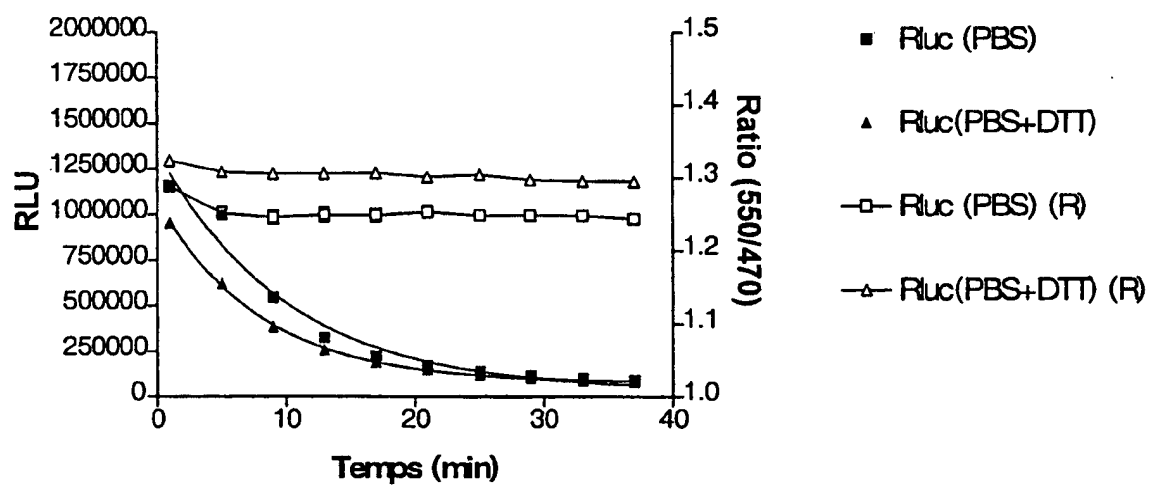


FIGURE 56

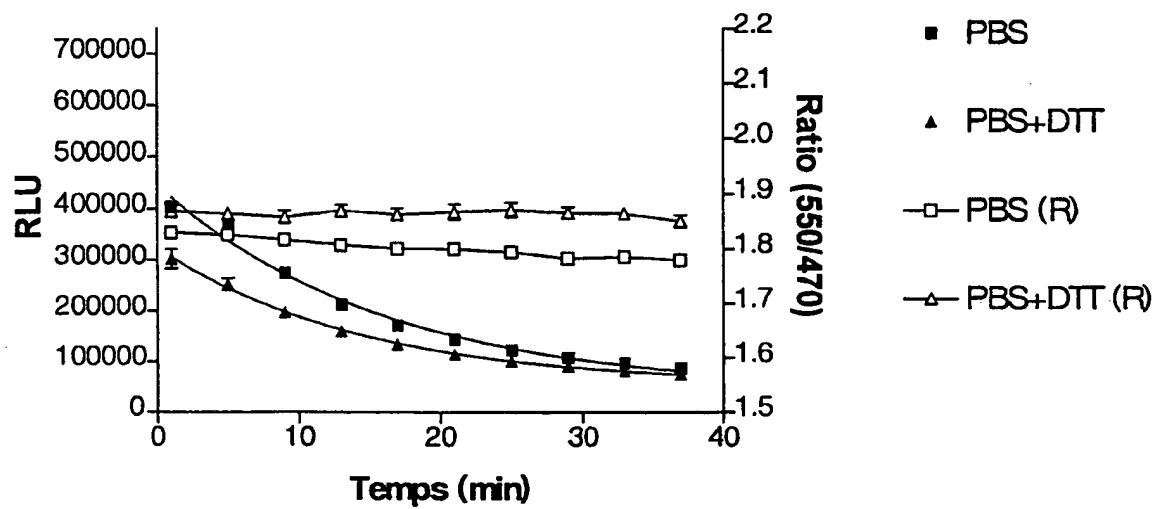


FIGURE 57

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ATGACTTCGA AAGTTTATGA TCCAGAACAA AGGAAACGGA TGATAACTGG TCCGCAGTGG
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AAACATGCAG AAAATGCTGT TATTTTTTTA CATGGTAACG CGGCCTCTTC TTATTTATGG
CGACATGTTG TGCCACATAT TGAGCCAGTA GCGCGGTGTA TTATACCAGA CCTTATTGGT
ATGGGCAAAT CAGGCAAATC TGGTAATGGT TCTTATAGGT TACTTGATCA TTACAAATAT
CTTACTGCAT GGTTTGAAC TCTTAATTTA CCAAAGAAGA TCATTTTTGT CGGCCATGAT
TGGGGTGCTT GTTTGGCATT TCATTATAGC TATGAGCATC AAGATAAGAT CAAAGCAATA
GTTACGCTG AAAGTGTAGT AGATGTGATT GAATCATGGG ATGAATGGCC TGATATTGAA
GAAGATATTG CGTTGATCAA ATCTGAAGAA GGAGAAAAAA TGGTTTTGGA GAATAACTTC
TTCGTGGAAA CCATGTTGCC ATCAAAAATC ATGAGAAAAGT TAGAACCAGA AGAATTTGCA
GCATATCTTG AACCATTCAA AGAGAAAGGT GAAGTTCGTC GTCCAACATT ATCATGGCCT
CGTGAAATCC CGTTAGTAAA AGGTGGTAAA CCTGACGTTG TACAAATTGT TAGGAATTAT
AATGCTTATC TACGTGCAAG TGATGATTTA CCAAAAATGT TTATTGAATC GGACCCAGGA
TTCTTTTCCA ATGCTATTGT TGAAGGTGCC AAGAAGTTTC CTAATACTGA ATTTGTCAAA
GTAAAAGGTC TTCATTTTTT GCAAGAAGAT GCACCTGATG AAATGGGAAA ATATATCAAA
TCGTTCGTTG AGCGAGTTCT CAAAAATGAA CAACGGGCCC GGGATCCCCG GGTACCGGTC
GCCACCATGG TGAGCAAGGG CGAGGAGCTG TTCACCGGGG TGGTGCCCAT CCTGGTCGAG
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ACCTACGGCA AGCTGACCCT GAAGTTCATC TGCACCACCG GCAAGCTGCC CGTGCCCTGG
CCCACCCTCG TGACCACCTT CGGCTACGGC CTGCAGTGCT TCGCCCGCTA CCCCACCAC
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ATCTTCTTCA AGGACGACGG CAACTACAAG ACCCGCGCCG AGGTGAAGTT CGAGGGCGAC
ACCTGGTGTA ACCGCATCGA GCTGAAGGGC ATCGACTTCA AGGAGGACGG CAACATCCTG
GGGCACAAGC TGGAGTACAA CTACAACAGC CACAACGTCT ATATCATGGC CGACAAGCAG
AAGAACGGCA TCAAGGTGAA CTTCAAGATC CGCCACAACA TCGAGGACGG CAGCGTGCAG
CTCGCCGACC ACTACCAGCA GAACACCCCC ATCGGCGACG GCCCCGTGCT GCTGCCCGAC
AACCCTACC TGAGCTACCA GTCCGCCCTG AGCAAAGACC CCAACGAGAA GCGCGATCAC
ATGGTCCTGC TGGAGTTCGT GACCGCCGCC GGGATCACTC TCGGCATGGA CGAGCTGTAC
AAGTAA

```



FIGURE 58

ATGACTTCGA	AAGTTTATGA	TCCAGAACAA	AGGAAACGGA	TGATAACTGG	TCCGCAGTGG
TGGGCCAGAT	GTAAACAAAT	GAATGTTCTT	GATTCATTTA	TTAATTATTA	TGATTCAGAA
AAACATGCAG	AAAATGCTGT	TATTTTTTTA	CATGGTAACG	CGGCCTCTTC	TTATTTATGG
CGACATGTTG	TGCCACATAT	TGAGCCAGTA	GCGCGGTGTA	TTATACCAGA	CCTTATTGGT
ATGGGCAAAT	CAGGCAAATC	TGGTAATGGT	TCTTATAGGT	TACTTGATCA	TTACAAATAT
CTTACTGCAT	GGTTTGAAC	TCTTAATTTA	CCAAAGAAGA	TCATTTTGT	CGGCCATGAT
TGGGGTGCTT	GTTTGGCATT	TCATTATAGC	TATGAGCATC	AAGATAAGAT	CAAAGCAATA
G TTCACGCTG	AAAGTG TAGT	AGATGTGATT	GAATCATGGG	ATGAATGGCC	TGATATTGAA
GAAGATATTG	CGTTGATCAA	ATCTGAAGAA	GGAGAAAAAA	TGGTTTTGGA	GAATAACTTC
TTCGTGGAAA	CCATGTTGCC	ATCAAAAATC	ATGAGAAAGT	TAGAACCAGA	AGAATTTGCA
GCATATCTTG	AACCATTCAA	AGAGAAAGGT	GAAGTTCGTC	GTCCAACATT	ATCATGGCCT
CGTGAAATCC	CGTTAGTAAA	AGGTGGTAAA	CCTGACGTTG	TACAAATTGT	TAGGAATTAT
AATGCTTATC	TACGTGCAAG	TGATGATTTA	CCAAAAATGT	TTATTGAATC	GGACCCAGGA
TTCTTTTCCA	ATGCTATTGT	TGAAGGTGCC	AAGAAGTTTC	CTAATACTGA	ATTTGTCAAA
GTAAAAGGTC	TTCATTTTTTC	GCAAGAAGAT	GCACCTGATG	AAATGGGAAA	ATATATCAAA
TCGTTTCGTTG	AGCGAGTTCT	CAAAAATGAA	CAACGGGCC	GGGATCCGGG	CGACGATGAC
GATAAGTTGG	CGGTACCGGT	CGCCACCATG	GTGAGCAAGG	GCGAGGAGCT	G TTCACCGGG
GTGGTGCCCA	TCCTGGTCGA	GCTGGACGGC	GACGTAAACG	GCCACAAGTT	CAGCGTGTCC
GGCGAGGGCG	AGGGCGATGC	CACCTACGGC	AAGCTGACCC	TGAAGTTCAT	CTGCACCACC
GGCAAGCTGC	CCGTGCCCTG	GCCCACCCTC	GTGACCACCT	TCGGCTACGG	CCTGCAGTGC
TTCGCCCCTG	ACCCCGACCA	CATGAAGCAG	CACGACTTCT	TCAAGTCCGC	CATGCCCCGAA
GGCTACGTCC	AGGAGCGCAC	CATCTTCTTC	AAGGACGACG	GCAACTACAA	GACCCGCGCC
GAGGTGAAGT	TCGAGGGCGA	CACCCTGGTG	AACCGCATCG	AGCTGAAGGG	CATCGACTTC
AAGGAGGACG	GCAACATCCT	GGGGCACAAG	CTGGAGTACA	ACTACAACAG	CCACAACGTC
TATATCATGG	CCGACAAGCA	GAAGAACGGC	ATCAAGGTGA	ACTTCAAGAT	CCGCCACAAC
ATCGAGGACG	GCAGCGTGCA	GCTCGCCGAC	CACTACCAGC	AGAACACCCC	CATCGGCGAC
GGCCCCGTGC	TGCTGCCCCG	CAACCACTAC	CTGAGCTACC	AGTCCGCCCT	GAGCAAAGAC
CCCAACGAGA	AGCGCGATCA	CATGGTCCTG	CTGGAGTTTC	TGACCGCCGC	CGGGATCACT
CTCGGCATGG	ACGAGCTGTA	CAAGTAAA			

FIGURE 59

ATGACTTCGA	AAGTTTATGA	TCCAGAACAA	AGGAAACGGA	TGATAACTGG	TCCGCAGTGG
TGGGCCAGAT	GTAAACAAAT	GAATGTTCTT	GATTCATTTA	TTAATTATTA	TGATTCAGAA
AAACATGCAG	AAAATGCTGT	TATTTTTTTA	CATGGTAACG	CGGCCTCTTC	TTATTTATGG
CGACATGTTG	TGCCACATAT	TGAGCCAGTA	GCGCGGTGTA	TTATACCAGA	CCTTATTGGT
ATGGGCAAAT	CAGGCAAATC	TGGTAATGGT	TCTTATAGGT	TACTTGATCA	TTACAAATAT
CTTACTGCAT	GGTTTGAAC	TCTTAATTTA	CCAAAGAAGA	TCATTTTTGT	CGGCCATGAT
TGGGGTGCTT	GTTTGGCATT	TCATTATAGC	TATGAGCATC	AAGATAAGAT	CAAAGCAATA
GTTACGCTG	AAAGTGTAGT	AGATGTGATT	GAATCATGGG	ATGAATGGCC	TGATATTGAA
GAAGATATTG	CGTTGATCAA	ATCTGAAGAA	GGAGAAAAAA	TGGTTTTGGA	GAATAACTTC
TTCGTGGAAA	CCATGTTGCC	ATCAAAAATC	ATGAGAAAGT	TAGAACCAGA	AGAATTTGCA
GCATATCTTG	AACCATTCAA	AGAGAAAGGT	GAAGTTCGTC	GTCCAACATT	ATCATGGCCT
CGTGAAATCC	CGTTAGTAAA	AGGTGGTAAA	CCTGACGTTG	TACAAATTGT	TAGGAATTAT
AATGCTTATC	TACGTGCAAG	TGATGATTTA	CCAAAAATGT	TTATTGAATC	GGACCCAGGA
TTCTTTTCCA	ATGCTATTGT	TGAAGGTGCC	AAGAAGTTTC	CTAATACTGA	ATTTGTCAAA
GTAAAAGGTC	TTCATTTTTT	GCAAGAAGAT	GCACCTGATG	AAATGGGAAA	ATATATCAAA
TCGTTCGTTG	AGCGAGTTCT	CAAAAATGAA	CAACGGGCCC	GGGATCCGGC	CGACGAGGTG
GACGGCGAAT	CCGCGGTACC	GGTCGCCACC	ATGGTGAGCA	AGGGCGAGGA	GCTGTTCAAC
GGGGTGGTGC	CCATCCTGGT	CGAGCTGGAC	GGCGACGTAA	ACGGCCACAA	GTTCAGCGTG
TCCGGCGAGG	GCGAGGGCGA	TGCCACCTAC	GGCAAGCTGA	CCCTGAAGTT	CATCTGCACC
ACCGGCAAGC	TGCCCCTGCC	CTGGCCCACC	CTCGTGACCA	CCTTCGGCTA	CGGCCTGCAG
TGCTTCGCCC	GCTACCCCGA	CCACATGAAG	CAGCACGACT	TCTTCAAGTC	CGCCATGCCC
GAAGGCTACG	TCCAGGAGCG	CACCATCTTC	TTCAAGGACG	ACGGCAACTA	CAAGACCCGC
GCCGAGGTGA	AGTTCGAGGG	CGACACCCTG	GTGAACCGCA	TCGAGCTGAA	GGGCATCGAC
TTCAAGGAGG	ACGGCAACAT	CCTGGGGCAC	AAGCTGGAGT	ACAAC TACAA	CAGCCACAAC
GTCTATATCA	TGGCCGACAA	GCAGAAGAAC	GGCATCAAGG	TGAACTTCAA	GATCCGCCAC
AACATCGAGG	ACGGCAGCGT	GCAGCTCGCC	GACCACTACC	AGCAGAACAC	CCCCATCGGC
GACGGCCCCG	TGCTGCTGCC	CGACAACCAC	TACCTGAGCT	ACCAGTCCGC	CCTGAGCAAA
GACCCCAACG	AGAAGCGCGA	TCACATGGTC	CTGCTGGAGT	TCGTGACCGC	CGCCGGGATC
ACTCTCGGCA	TGGACGAGCT	GTACAAGTAA			

FIGURE 60

ATGGTGAGCA	AGGGCGAGGA	GCTGTTCACC	GGGGTGGTGC	CCATCCTGGT	CGAGCTGGAC
GGCGACGTAA	ACGGCCACAA	G TTCAGCGTG	TCCGGCGAGG	GCGAGGGCGA	TGCCACCTAC
GGCAAGCTGA	CCCTGAAGTT	CATCTGCACC	ACCGGCAAGC	TGCCCCGTGCC	CTGGCCCACC
CTCGTGACCA	CCTTCGGCTA	CGGCCTGCAG	TGCTTCGCCC	GCTACCCCGA	CCACATGAAG
CAGCACGACT	TCTTCAAGTC	CGCCATGCCC	GAAGGCTACG	TCCAGGAGCG	CACCATCTTC
TTCAAGGACG	ACGGCAACTA	CAAGACCCGC	GCCGAGGTGA	AGTTCGAGGG	CGACACCTTG
GTGAACCGCA	TCGAGCTGAA	GGGCATCGAC	TTCAAGGAGG	ACGGCAACAT	CCTGGGGCAC
AAGCTGGAGT	ACAACTACAA	CAGCCACAAC	GTCTATATCA	TGGCCGACAA	GCAGAAGAAC
GGCATCAAGG	TGAACTTCAA	GATCCGCCAC	AACATCGAGG	ACGGCAGCGT	GCAGCTCGCC
GACCACTACC	AGCAGAACAC	CCCCATCGGC	GACGGCCCCG	TGCTGCTGCC	CGACAACCAC
TACCTGAGCT	ACCAGTCCGC	CCTGAGCAAA	GACCCCAACG	AGAAGCGCGA	TCACATGGTC
CTGCTGGAGT	TCGTGACCGC	CGCCGGGATC	ACTCTCGGCA	TGGACGAGCT	GTACAAGAGA
TCTATGACCA	TGGACTCTGG	AGCAGACAAC	CAGCAGAGTG	GAGATGCTGC	TGTAACAGAA
GCTGAAAGTC	AACAAATGAC	AGTTCAAGCC	CAGCCACAGA	TTGCCACATT	AGCCCAGGTA
TCCATGCCAG	CAGCTCATGC	GACGTCATCT	GCTCCCCTG	TAACCTTAGT	GCAGCTGCCC
AATGGGCAGA	CAGTCCAGGT	CCATGGGGTC	ATCCAGGCGG	CCCAGCCATC	AGTTATTCAG
TCTCCACAAG	TCCAAACAGT	TCAGTCTTCC	TGTAAGGACT	TAAAAAGACT	TTTCTCCGGA
ACTCAGATTT	CAACTATTGC	AGAAAGTGAA	GATTCACAGG	AGTCTGTGGA	TAGTGTAAC
GATTCCCAA	AACGAAGGGA	AATCCTTTCA	AGGAGGCCCT	CCTACAGGAA	AATTTTGAAT
GACTTATCTT	CTGATGCACC	AGGGGTGCCA	AGGATTGAAG	AAGAAAAATC	AGAAGAAGAG
ACTTCAGCCC	CTGCCATCAC	CACTGTAACA	GTGCCAACCC	CGATTTACCA	AACTAGCAGT
GGGCAGTATA	TTGCCATTAC	CCAGGGAGGA	GCAATACAGC	TGGCTAACAA	TGGTACCGAT
GGGGTACAGG	GCCTGCAGAC	ATTAACCATG	ACCAATGCAG	CTGCCACTCA	GCCGGGTACT
ACCATTCTAC	AATATGCACA	GACCACTGAT	GGACAGCAGA	TTCTAGTGCC	CAGCAACCAA
GTTGTTGTTT	AAGCTGCCTC	TGGTGATGTA	CAAACATACC	AGATTTCGCAC	AGCACCCACT
AGCACCATTG	CCCCTGGAGT	TGTTATGGCG	TCCTCCCCAG	CACTTCCTAC	ACAGCCTGCT
GAAGAAGCAG	CAAAGCTTGG	TACCGAGCTC	GGATCCCCCA	AGAAGAAGAG	GAAGGTGTGA

## FIGURE 61

ATGACTTCGA	AAGTTTATGA	TCCAGAACAA	AGGAAACGGA	TGATAACTGG	TCCGCAGTGG
TGGGCCAGAT	GTAAACAAAT	GAATGTTCTT	GATTCATTTA	TTAATTATTA	TGATTCAGAA
AAACATGCAG	AAAATGCTGT	TATTTTTTTA	CATGGTAACG	CGGCCTCTTC	TTATTTATGG
CGACATGTTG	TGCCACATAT	TGAGCCAGTA	GCGCGGTGTA	TTATACCAGA	CCTTATTGGT
ATGGGCAAAT	CAGGCAAATC	TGGTAATGGT	TCTTATAGGT	TACTTGATCA	TTACAAATAT
CTTACTGCAT	GGTTTGAAC	TCTTAATTTA	CCAAAGAAGA	TCATTTTTGT	CGGCCATGAT
TGGGGTGCTT	GTTTGGCATT	TCATTATAGC	TATGAGCATC	AAGATAAGAT	CAAAGCAATA
GTTCACGCTG	AAAGTGTAGT	AGATGTGATT	GAATCATGGG	ATGAATGGCC	TGATATTGAA
GAAGATATTG	CGTTGATCAA	ATCTGAAGAA	GGAGAAAAAA	TGGTTTTGGA	GAATAACTTC
TTCGTGGAAA	CCATGTTGCC	ATCAAAAATC	ATGAGAAAGT	TAGAACCAGA	AGAATTTGCA
GCATATCTTG	AACCATTCAA	AGAGAAAGGT	GAAGTTCGTC	GTCCAACATT	ATCATGGCCT
CGTGAAATCC	CGTTAGTAAA	AGGTGGTAAA	CCTGACGTTG	TACAAATTGT	TAGGAATTAT
AATGCTTATC	TACGTGCAAG	TGATGATTTA	CCAAAAATGT	TTATTGAATC	GGACCCAGGA
TTCTTTTCCA	ATGCTATTGT	TGAAGGTGCC	AAGAAGTTTC	CTAATACTGA	ATTTGTCAAA
GTAAAAGGTC	TTCATTTTTT	GCAAGAAGAT	GCACCTGATG	AAATGGGAAA	ATATATCAAA
TCGTTCGTTG	AGCGAGTTCT	CAAAAATGAA	CAACGGGCCC	GGGATCCCCG	GGTACCGACA
GGGCAACAGA	ATGCCACTTC	TTTAAGTAAC	CCAAATCCCA	TAGACCCCAG	CTCCATGCAG
CGAGCCTATG	CTGCTCTCGG	ACTCCCCTAC	ATGAACCAGC	CCCAGACGCA	GCTGCAGCCT
CAGGTTTCCTG	GCCAGCAACC	AGCACAGCCT	CAAACCCACC	AGCAGATGAG	GACTCTCAAC
CCCCTGGGAA	ATAATCCAAT	GAACATTCCA	GCAGGAGGAA	TAACAACAGA	TCAGCAGCCC
CCAAACTTGA	TTTCAGAATC	AGCTCTTCCG	ACTTCCCTGG	GGGCCACAAA	CCCCTGATG
AACGATGGCT	CCAACCTCTG	TAACATTGGA	ACCCTCAGCA	CTATACCAAC	AGCAGCTCCT
CCTTCTAGCA	CCGGTGTAAG	GAAAGGCTGG	CACGAACATG	TCACTCAGGA	CCTGCGGAGC
CATCTAGTGC	ATAAACTCGT	CCAAGCCATC	TTCCCAACAC	CTGATCCCGC	AGCTCTAAAG
GATCGCCGCA	TGGAAAACCT	GGTAGCCTAT	GCTAAGAAAG	TGGAAGGGGA	CATGTACGAG
TCTGCCAACA	GCAGGGATGA	ATATTATCAC	TTATTAGCAG	AGAAAATCTA	CAAGATAGCG
GCCGCTCGAG	AATTCCCCAA	GAAGAAGAGG	AAGGTGTAG		

## FIGURE 62

ATGGTGAGCA	AGGGCGAGGA	GCTGTTACAC	GGGGTGGTGC	CCATCCTGGT	CGAGCTGGAC
GGCGACGTAA	ACGGCCACAA	GTTCAGCGTG	TCCGGCGAGG	GCGAGGGCGA	TGCCACCTAC
GGCAAGCTGA	CCCTGAAGTT	CATCTGCACC	ACCGGCAAGC	TGCCCCGTGCC	CTGGCCCCACC
CTCGTGACCA	CCTTCGGCTA	CGGCCTGCAG	TGCTTCGCCC	GCTACCCCGA	CCACATGAAG
CAGCACGACT	TCTTCAAGTC	CGCCATGCCC	GAAGGCTACG	TCCAGGAGCG	CACCATCTTC
TTCAAGGACG	ACGGCAACTA	CAAGACCCGC	GCCGAGGTGA	AGTTCGAGGG	CGACACCCTG
GTGAACCGCA	TCGAGCTGAA	GGGCATCGAC	TTCAAGGAGG	ACGGCAACAT	CCTGGGGCAC
AAGCTGGAGT	ACAACTACAA	CAGCCACAAC	GTCTATATCA	TGGCCGACAA	GCAGAAGAAC
GGCATCAAGG	TGAACTTCAA	GATCCGCCAC	AACATCGAGG	ACGGCAGCGT	GCAGCTCGCC
GACCACTACC	AGCAGAACAC	CCCCATCGGC	GACGGCCCCG	TGCTGCTGCC	CGACAACCAC
TACCTGAGCT	ACCAGTCCGC	CCTGAGCAAA	GACCCCAACG	AGAAGCGCGA	TCACATGGTC
CTGCTGGAGT	TCGTGACCGC	CGCCGGGATC	ACTCTCGGCA	TGGACGAGCT	GTACAAGAGA
TCTCCCAAGA	ACTACATAGA	AATGAAACCA	CATCCGTGGT	TTTTTGCAA	AATCCCCAGA
GCCAAGGCAG	AAGAAATGCT	TAGCAAACAG	CGGCACGATG	GGGCCTTTCT	TATCCGAGAG
AGTGAGAGCG	CTCCTGGGGA	CTTCTCCCTC	TCTGTCAAGT	TTGGAAACGA	TGTGCAGCAC
TTCAAGGTGC	TCCGAGATGG	AGCCGGGAAG	TACTTCCTCT	GGGTGGTGAA	GTTCAATTCT
TTGAATGAGC	TGGTGGATTA	TCACAGATCT	ACATCTGTCT	CCAGAAACCA	GCAGATATTC
CTGCGGGACA	TAGAACAGGT	GCCACAGCAG	CCGACATACG	TCCAGGCCCT	CTGA

FIGURE 63

ATGACTTCGA	AAGTTTATGA	TCCAGAACAA	AGGAAACGGA	TGATAACTGG	TCCGCAGTGG
TGGGCCAGAT	GTAAACAAAT	GAATGTTCTT	GATTCATTTA	TTAATTATTA	TGATTCAGAA
AAACATGCAG	AAAATGCTGT	TATTTTTTTA	CATGGTAACG	CGGCCTCTTC	TTATTTATGG
CGACATGTTG	TGCCACATAT	TGAGCCAGTA	GCGCGGTGTA	TTATACCAGA	CCTTATTGGT
ATGGGCAAAT	CAGGCAAATC	TGGTAATGGT	TCTTATAGGT	TACTTGATCA	TTACAAATAT
CTTACTGCAT	GGTTTGAAC	TCTTAATTTA	CCAAAGAAGA	TCATTTTTGT	CGGCCATGAT
TGGGGTGCTT	GTTTGGCATT	TCATTATAGC	TATGAGCATC	AAGATAAGAT	CAAAGCAATA
GTTACGCTG	AAAGTGTAGT	AGATGTGATT	GAATCATGGG	ATGAATGGCC	TGATATTGAA
GAAGATATTG	CGTTGATCAA	ATCTGAAGAA	GGAGAAAAAA	TGGTTTTGGA	GAATAACTTC
TTCGTGGAAA	CCATGTTGCC	ATCAAAAATC	ATGAGAAAGT	TAGAACCAGA	AGAATTTGCA
GCATATCTTG	AACCATTCAA	AGAGAAAGGT	GAAGTTCGTC	GTCCAACATT	ATCATGGCCT
CGTGAAATCC	CGTTAGTAAA	AGGTGGTAAA	CCTGACGTTG	TACAAATTGT	TAGGAATTAT
AATGCTTATC	TACGTGCAAG	TGATGATTTA	CCAAAAATGT	TTATTGAATC	GGACCCAGGA
TTCTTTTCCA	ATGCTATTGT	TGAAGGTGCC	AAGAAGTTTC	CTAATACTGA	ATTTGTCAAA
GTAAAAGGTC	TTCATTTTTTC	GCAAGAAGAT	GCACCTGATG	AAATGGGAAA	ATATATCAAA
TCGTTCTGTTG	AGCGAGTTCT	CAAAAATGAA	CAACGGGCC	GGGATCCCCG	GGTACCGATG
AACAAGCTGA	GTGGAGGCGG	CGGGCGCAGG	ACTCGGGTGG	AAGGGGGCCA	GCTTGGGGGC
GAGGAGTGGA	CCCGCCACGG	GAGCTTTGTC	AATAAGCCCA	CGCGGGGCTG	GCTGCATCCC
AACGACAAAG	TCATGGGACC	CGGGGTTTCC	TACTTGGTTC	GGTACATGGG	TTGTGTGGAG
GTCCTCCAGT	CAATGCGTGC	CCTGGACTTC	AACACCCGGA	CTCAGGTCAC	CAGGGAGGCC
ATCAGTCTGG	TGTGTGAGGC	TGTGCCGGGT	GCTAAGGGGG	CGACAAGGAG	GAGAAAGCCC
TGTAGCCGCC	CGCTCAGCTC	TATCCTGGGG	AGGAGTAACC	TGAAATTTGC	TGGAATGCCA
ATCACTCTCA	CCGTCTCCAC	CAGCAGCCTC	AACCTCATGG	CCGCAGACTG	CAAACAGATC
ATCGCCAACC	ACCACATGCA	ATCTATCTCA	TTTGCATCCG	GCGGGGATCC	GGACACAGCC
GAGTATGTCTG	CCTATGTTGC	CAAAGACCCT	GTGAATCAGA	GAGCCTGCCA	CATTCTGGAG
TGTCCCGAAG	GGCTTGCCCA	GGATGTCATC	AGCACCATTG	GCCAGGCCTT	CGAGTTGCGC
TTCAAACAAT	ACCTCAGGAA	CCCACCCAAA	CTGGTCACCC	CTCATGACAG	GATGGCTGGC
TTTGATGGCT	CAGCATGGGA	TGAGGAGGAG	GAAGAGCCAC	CTGACCATCA	GTACTATAAT
GACTTCCCGG	GGAAGGAACC	CCCCTTGGGG	GGGGTGGTAG	ACATGAGGCT	TCGGGAAGGA
GCCGCTCCAG	GGGCTGCTCG	ACCCACTGCA	CCCAATGCC	AGACCCCAG	CCACTTGGGA
GCTACATTGC	CTGTAGGACA	GCCTGTTGGG	GGAGATCCAG	AAGTCCGCAA	ACAGATGCCA
CCTCCACCAC	CCTGTCCAGG	CAGAGAGCTT	TTTGATGATC	CCTCCTATGT	CAACGTCCAG
AACCTAGACA	AGGCCCGGCA	AGCAGTGGGT	GGTGCTGGGC	CCCCAATCC	TGCTATCAAT
GGCAGTGCAC	CCCGGGACCT	GTTTGACATG	AAGCCCTTCG	AAGATGCTCT	TCGGGTGCCT
CCACCTCCCC	AGTCGGTGTC	CATGGCTGAG	CAGCTCCGAG	GGGAGCCCTG	A